

being designated Claim 53. The Examiner corrected that error by renumbering the second claim 53, the composition claim, as claim 54 under 37 C.F.R. 1.126. As a consequence, Applicants have amended claims 50, 51, 52 and 53 to depend from said composition claim 54.

The Examiner also noted that claims 44-46 were incomplete in depending from cancelled claim 43. Applicants have corrected said oversight in amending claims 44-46 to depend from claim 54. Applicants submit that such an amendment overcomes the Examiner's rejection of claims 44-46 under §112, second paragraph.

Reconsideration of the above-identified patent application is respectfully requested in view of the above amendments and for the reasons that follow.

#### Background

By way of background review, the instantly claimed invention relates to modified human fibroblast interferons which have the biological activity of normal human fibroblast interferon. Specifically, Applicants have discovered that by deleting or substituting the cysteine residue at position 17 with a neutral amino acid, the resulting modified protein still retains normal fibroblast interferon activity. This result would not be anticipated because 1) it is known that simple single amino acid substitution or modifications of peptides or proteins have the potential of dramatically altering or removing biological activity and 2) Shepard et al., Nature, 294, 563 (1981), of record, shows that substitution of the cysteine at position 141 of beta-interferon (IFN- $\beta$ ) with tyrosine destroys IFN- $\beta$ 's activity. Thus, the Shepard et al. article and analogous references relating to other proteins, point to the novelty and unobviousness of Applicants' invention.

Enclosure 1 is an article from McGraw-Hill's Biotechnology Newswatch, Volume 3, Number 11, June 6, 1983 relative to the subject matter claimed in the instant patent application. The article points out that Cetus' IFN- $\beta$  mutein has greater stability and about ten times the specific activity of earlier recombinant IFN- $\beta$ .

The article goes on to state:

Prior animal and in vitro testing of the modified interferon indicates that it is very similar to the native molecule in its antiviral and immunological activity. It also appears to be nontoxic to animals....[T]he key to Cetus' creation of its beta-interferon variant lay in formulating and testing a hypothesis that there is a link between the molecule's disulfide chemistry and its degree of specific activity and stability. Human fibroblast (beta) interferon has three cysteine residues at key positions, only two of which are involved in forming an intramolecular disulfide bond. Thus the third cysteine residue can form covalent dimers with other interferon molecules, leading to a decrease in potency.

(Emphasis added.)

The following comments found at page 2 attributed to Dr. Ron Wetzel of Genentech, Inc. regarding the instant invention are noteworthy:

Across the bay in South San Francisco, Ron Wetzel, a senior scientist at Genentech, Inc., calls David Mark's result 'quite exciting.' It is, he says, 'the first example of a practical application of site-specific mutagenesis used on a rational basis to make useful improvements in a protein's properties.'

Wetzel adds that though many people have experimented with site-specific mutagenesis, Cetus' result represents the 'first really significant practical accomplishment in this area.'

The fact that the mutant IFN- $\beta$  proved to have a longer shelf-life is significant. As indicated at the end of the article, another company had to withdraw its recombinant IFN- $\beta$  product from clinical trials because of a "question of stability."

#### 101 and 112 Rejections Under 35 U.S.C. §§101 and 112, Utility

Claims 44-46 and 50-54 stand rejected under 35 USC §101 and §112, first paragraph, for insufficient proof of utility. Applicants observe that claim 47 to "biologically active IFN- $\beta_{Ser17}$ " and claim 49 to "IFN- $\beta_{Ser17}$  as represented by [its] amino acid sequence" have escaped this rejection. Applicants therefore infer that the Examiner

considers that utility has been sufficiently proven for IFN- $\beta_{\text{Ser17}}$  by the test results in the specification at pages 27-30.

Applicants respectfully submit that the utility of IFN- $\beta_{\text{Ser17}}$  by analogy proves the utility of the other claimed IFN- $\beta$  position 17 muteins. Enclosure 2 graphically illustrates the concept underlying the logic of the immediately preceding statement and is at the essence of the instant invention. That concept is described at page 7, lines 5-24 and touched on throughout the specification. In brief, the concept is that the cysteine at position 17 (cys 17) of recombinant IFN- $\beta$  is nonessential, and its sulfhydryl group interferes with the formation of an essential disulfide bond and contributes significantly to the formation of undesirable IFN- $\beta$  multimers.

Enclosure 2 shows that recombinant IFN- $\beta$  has three cysteine residues at positions 17, 31 and 141. Each cysteine because of its free sulfhydryl group (each such group represented by a hook in the picture of Enclosure 2) can form intramolecular or intermolecular disulfide bonds with other sulfhydryl-containing amino acids. Enclosure 2 represents the intramolecular situation.

Based on the homology between IFN- $\beta$  and IFN- $\alpha$ s, it was hypothesized that the disulfide bond between the cysteine at position 141 (cys 141) and that at position 31 (cys 31) was essential for biological activity. "By either deleting cys 17 or substituting it by a different amino acid, one can determine whether cys 17 is essential to biological activity, and its role in --SS-- bond formation." (Page 7, lines 15-19.) Substitution of cys 17 was done as outlined in the specification to form IFN- $\beta_{\text{Ser17}}$ .

The purified IFN- $\beta_{\text{Ser17}}$  was shown to have comparable antiviral and antiproliferative activity to that of naturally produced, native IFN- $\beta$  (pages 27-28). Thus, as hypothesized at page 7, lines 19-24, cys 17 was shown to be nonessential for the biological activity of the protein.

The Shepard et al. article which stands for the proposition that cys 141 is essential to the biological activity of the molecule, supports the concept portrayed in Enclosure 2. In Shepard et al. cys

141 was replaced by tyrosine 141 resulting in an inactive protein.

As cys 141 is essential for the protein's biological activity whereas cys 17 is not essential to activity, it can be deducted that it is the disulfide bond between cys 141 and cys 31 that is critical. By deleting cys 17 or replacing it with a neutral amino acid, that is one without a free sulphydryl group, the disulfide bond between cys 31 and cys 141 is more likely to form, and there is a limited chance for a free sulphydryl group to form undesirable intermolecular links which create dimers and oligomers rendering purification and separation of microbially produced IFN- $\beta$  very laborious and time consuming.

It is the elimination or substitution of cys 17 with a neutral amino acid that allows the recombinant mutein to achieve a proper, biologically active conformation. Thus, applicants respectfully submit that the claims of the instant application properly define the scope of the invention. It is the elimination of the sulphydryl group at position 17 which is the key to the invention and not what neutral amino acid happens to replace cys 17.

Further, Applicants submit that Shepard et al., discussed at page 2, lines 23-34 and at page 7, lines 7-9 of the specification, supports rather than casts doubt on the range of the IFN- $\beta$  position 17 mutesins claimed in the instant application. Enclosure 2 also illustrates why the Shepard et al. INF- $\beta$ <sub>tyr141</sub> mutein was ineffective. By eliminating the cys 141 sulphydryl group, the essential disulfide bond between cys 141 and cys 31 was unable to form resulting in an inactive product.

It is also respectfully submitted that the specification contains a detailed description of the methods of carrying out site-directed mutagenesis to prepare a wide variety of mutesins within the scope of the claims. Using available enzymes, reagents, plasmids and the host strains disclosed and deposited, those skilled in the art would be enabled to prepare the mutesins instantly claimed with a great deal of predictability and without undue experimentation.

In vivo versus In vitro

The Examiner further states regarding the §§101 and 102 rejection for utility that "there is no evidence in the record of any correlation between the in vitro activity disclosed in the specification with activity in humans." Applicants respectfully submit that the in vitro tests outlined in pages 27-30 are standard tests that are accepted in the art and would be understood by those of ordinary skill in the art to predict some degree of parallel activity in vivo.

For example, the test showing that IFN- $\beta$ <sub>Ser17</sub> significantly inhibited the growth of T24 cells derived from transitional cell carcinoma (page 28 of the specification, first full paragraph) was reviewed in the article enclosed (Enclosure 3) by Bradley et al. ["The human tumor colony-forming chemosensitivity assay: a biological and clinical review," Investigational New Drugs, 2, 59-70 (1984).] The Bradley et al. article shows that there is a very high correlation between "antiproliferative activity" in vitro test results (discussed as the CFCA test in the Bradley et al. article; see page 61, column 2) and clinical in vivo test results.

Stewart, W.E., II, The Interferon System (Springer-Verlag 1981) describes in Chapter II a number of interferon assays (pp. 13-25). Enclosure 4 is from that chapter (pages 17 and 18) which describes Cytopathic Effect (CPE)--Inhibition Assays. The CPE-Reading Method was that used to determine antiviral activity in the tests recorded in the specification at pages 27-28 showing that IFN- $\beta$ <sub>Ser17</sub> and naturally produced IFN- $\beta$  had comparable antiviral activity against vesicular stomatitis virus and herpes simplex virus type 1. Enclosure 4 indicates that the CPE-reading method "was first used by Ho and Enders" in 1959 "and has been used to assay nearly every type of interferon that has been described, against a great number of viruses...." (Enclosure 4, page 17). Applicants respectfully submit that such long time usage by those skilled in the art evidences that the test is standard and accepted in the art.

Further, Chapter XIV of the Stewart book, entitled "Interferon in the Clinic" outlines studies up to the date of

publication of that book showing clinical responses to various interferons. Enclosure 5 is a photocopy of Table 18 from that chapter (pages 308-310). As highlighted in the text immediately following Table 18 (at page 310), "human fibroblast interferon was also found to be effective in preventing vaccinia 'takes' (Scott et al., 1977c) and crude human fibroblast and human leukocyte interferon preparations seemed of similar efficacy (Scott et al., 1977b)."

Applicants submit the following logic:

1. the in vitro tests with the IFN- $\beta_{\text{Ser17}}$  outlined in the specification are standard and accepted in the art;
2. IFN- $\beta_{\text{Ser17}}$  has been shown to have in vitro antiviral and antiproliferative activity comparable to naturally produced IFN- $\beta$ , (see specification, pages 27-30);
3. naturally produced IFN- $\beta$  has been shown to have antiviral and antiproliferative activities in vivo; therefore
4. it would be a reasonable assumption by those of ordinary skill in the art that IFN- $\beta_{\text{Ser17}}$  would also have antiviral and antiproliferative activity in vivo.

Once recombinant technology reached the point wherein sufficient quantities of pure IFN- $\beta$  became available for testing, reports of IFN- $\beta$ 's therapeutic utility started appearing. [See for example, Maeyer et al., editors, The Biology of the Interferon System, Proceedings of the International Meeting on the Biology of the Interferon System, held in Rotterdam, The Netherlands on 21-24 April, 1981, which contains the following articles: Gresser, I., Effects of Interferon In Vivo, pp. 141-147 (interferon shown to increase NK cell activity in mice and man and exert antitumor activity in experimental animals and man; also side effects mentioned); Niethammer et al., The Use of IFN- $\beta$  as an Antitumor Agent in Children, pp. 405-408 (discussion at p. 408 states: "Responses in adults have been observed in myeloma with IFN- $\beta$ ....").]

The Court of Appeals for the Federal Circuit has held that in vitro utility can be sufficient to comply with the practical

utility requirement of §101. Cross v. Iizuka, 224 USPQ 739 at 747 (CAFC 1985).

The CAFC states therein at page 747:

Our predecessor court has noted that adequate proof of any pharmacological activity constitutes a showing of practical utility.... Dr. Ramwell testified that initial testing of compounds for a particular pharmacological activity is typically done in vitro. In vitro testing permits an investigator to establish the rank order of compounds with respect to the particular pharmacological activity, i.e., to determine the relative potency of the compounds. Compounds having the highest ranking or potency are then selected for further testing in vivo.... In vitro testing, in general, is relatively less complex, less time consuming, and less expensive than in vivo testing. Moreover, in vitro results with respect to the particular pharmacological activity are generally predictive of in vivo test results, i.e., there is a reasonable correlation therebetween.... Iizuka has not urged, and rightly so, that there is an invariable exact correlation between in vitro test results and in vivo test results. Rather, Iizuka's position is that successful in vitro testing for a particular pharmacological activity establishes a significant probability that in vivo testing for this particular pharmacological activity will be successful.

[Emphasis added; cites omitted.]

The Board of Patent Interferences has also held that "a standard in vitro test may be sufficient to demonstrate pharmacological activity of a compound, i.e., 'practical utility.'" Bigham v. Godtfredsen and Von Daehne, 222 USPQ 632 at 637 (PBPI 1984).

The CCPA in In re Langer, 183 USPQ 288 (CCPA 1974) indicated that human testing is not always necessary to establish the utility of a claimed compound wherein its intended uses include human consumption.

As indicated at page 30 of the specification (lines 5-6), Phase I clinical trials to verify the safety of IFN- $\beta$ <sub>Ser17</sub> have been initiated. Since IFN- $\beta$ <sub>Ser17</sub> showed comparable biological activity to

IFN- $\beta$  in vitro, it can be predicted with reasonable certainty that IFN- $\beta$ <sub>Ser17</sub> will show comparable in vivo activity to IFN- $\beta$ . Therefore, Applicants respectfully submit that "there is a reasonable correlation" (Cross v. Iizuka, quote, supra) between in vitro and in vivo test results and expectation that the in vitro test results outlined in the specification would be predictive of in vivo test results.

The specification at pages 28-29 also indicates the ability of IFN- $\beta$ <sub>Ser17</sub> to stimulate NK cell (spontaneous cell mediated cytotoxicity).

The press report of Enclosure 1 evidences that the IFN- $\beta$  muteins have received wide-spread publicity and that there has been considerable investment and anticipation by those skilled in the art that the instant muteins will be successful therapeutics.

Applicants respectfully point out that the absence of in vivo data should not be critical. Applicants respectfully submit that it has long been settled that in the absence of any evidence or apparent reason why the claimed compounds do not possess the disclosed utility, the allegation of utility in the specification must be accepted as correct. In re Kamal and Rogier, 158 USPQ 320 (CCPA 1958) and In re Riat, Demontmollin, and Koller, 140 USPQ 471 (CCPA 1964).

The Examiner has given no reason for doubting the utilities of the instantly claimed muteins. Applicants respectfully submit that the disclosed utilities should be acceptable and the rejection withdrawn. In re Gazave, 154 USPQ 92 (CCPA 1967) and In re Bundy, 209 USPQ 48 (CCPA 1981).

The CAFC further held in Cross v. Iizuka, id. that the 35 USC 112 "how to use" requirement was satisfied in that case despite the failure of the disclosure to reveal dosages for the novel compound per se, in that, those skilled in the art had sufficient information at the critical date to determine dosage for desired pharmacological activity.

The specification at page 30, lines 5-11 indicates that Phase I clinical trials have begun testing IFN- $\beta$ <sub>Ser17</sub> by administering

intramuscularly and intravenously doses ranging between  $1 \times 10^5$  (1 $\mu$ g) to  $400 \times 10^6$  units of the protein. A variety of pharmaceutically acceptable formulations and modes of administration are suggested at pages 30-31. Applicants submit that the specification also satisfies the "how to use" requirement of §112 as interpreted in Cross v. Iizuka, supra.

Rejection Under 35 U.S.C §112, Enablement

The specification has been objected to under 35 USC 112, first paragraph, as failing to provide an enabling disclosure. The Examiner states: "Applicants' invention depends on the use of new plasmids and microorganisms that are not known nor readily available to the skilled artisan. In order to fully satisfy the requirements of the statute, a deposit of new microorganisms commensurate in scope with the claims is required." All the claims of the instant application, claims 44-47 and 49-54, are rejected as a consequence.

Applicants respectfully submit that the specification contains "a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same" and sets "forth the best mode contemplated by the inventor[s] of carrying out" their invention. Applicants argue that even without any deposits of microorganisms that the specification stands on its own as sufficiently enabling.

However, Applicants did make two deposits for the instant application. Plasmid pSY2501 was cloned into two E. coli host strains, MM294 and a subvariant strain thereof MM294-1. The specification at page 19, lines 3-10, notes that the former clone of pSY2501 [also designated CMCC (Cetus Master Culture Collection) No. 1533] was deposited with the Agricultural Research Culture Collection

(NRRL) on March 30, 1983 under accession number NRRL B-15356, and at page 20, lines 14-20, that the latter clone of pSY2501 [also designated CMCC No. 1494] was deposited with the American Type Culture Collection (ATCC) on November 18, 1983 under ATCC No. 39,517.

Enclosures 6(A) and 6(B) provide copies of the appropriate documentation outlining the correspondence between Cetus and respectively the NRRL and the ATCC in regard to said deposits and establishes that the deposits were made in compliance with the maintenance requirements of MPEP 608.01(p) part 3 and Rule 9.1 of the Budapest Treaty.

Plasmid pSY2501, for which a restriction map is provided in Figure 9 of the specification, contains a DNA sequence coding for the mutein of  $\beta$ -interferon (IFN- $\beta$ ) wherein the cysteine at position 17 is replaced by serine, that is, IFN- $\beta$ <sub>ser17</sub>.

The IFN- $\beta$ <sub>ser17</sub> mutein is representative of a variety of muteins that can be made and used according to the procedures outlined in the specification without undue experimentation. These muteins are described in Claim 54 as "[r]ecombinant, synthetic human interferon- $\beta$  muteins wherein the cysteine at position 17, numbered in accordance with native interferon- $\beta$ , is deleted or replaced by a neutral amino acid, and wherein said mutein exhibits biological activity of native, human interferon- $\beta$ ."

The specification states at page 3, lines 1-4, that "[d]irected mutagenesis techniques are well known and have been reviewed by Lather, R.F. and Lecoq, J.P. in Genetic Engineering Academic Press (1983), pp. 31-50. Oligonucleotide-directed mutagenesis is specifically reviewed by Smith, M. and Gillam, S. in Genetic Engineering: Principles and Methods, Plenum Press (1981) 3:1-32."

The method for making the synthetic IFN- $\beta$  mutein structural genes by oligonucleotide-directed mutagenesis is outlined at page 4, lines 4-20. Figure 2 is a schematic illustration showing the preparation of a mutant IFN- $\beta$  gene by oligonucleotide-directed mutagenesis. Pages 9-13 of the specification generally describe

oligonucleotide-directed mutagenesis as adapted to create the IFN- $\beta$  position 17 muteins of the instant invention.

Example 2 describes with great particularity a site-directed mutagenesis in the preparation of pSY2501. Examples 3 and 4 indicate respectively that IFN- $\beta$  threonine 17 and IFN- $\beta$  ~~cysteine deletion~~ <sup>cysteine deletion</sup> ~~alanine~~ 17 <sup>can be</sup> prepared by the procedure of Example 2 by appropriately substituting or deleting the codon for serine at position 17 of IFN- $\beta$  in synthesizing the oligonucleotide primer required for the site-directed mutagenesis. Exemplary primers are also shown at page 9, lines 30-33 and page 10, lines 1-4. The primers may be prepared by known methods, for example, the triester method [Itakura et al., (1975) J. Biol. Chem., 250, 4592-4600].

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To prepare any other desired IFN- $\beta$  position 17 muteins, one of ordinary skill in the art can easily refer to readily available tables matching codons with the amino acids for which they code, and then substitute the appropriate codon in the oligonucleotide primer as exemplified.

Example 1 describes the cloning of the IFN- $\beta$  gene into an M13 vector. Single-stranded phage DNA prepared by the procedures of Example 1 serve as a template for site-specific mutagenesis using the appropriately substituted or deleted synthetic oligonucleotide.

The appropriately substituted or deleted primer exemplified in the specification is synthesized and hybridized to single stranded M13 phage DNA, which carries the sense strand of the IFN- $\beta$  gene. (As noted at page 10, lines 11-24, the phage could have carried the antisense strand.)

The use of M13 phage vector as a source of single-stranded DNA template has been documented. Temple et al., Nature (London), 296, 537-540 (1982); Gillam et al., Gene 8, 81-97 (1979); Gillam et al., Gene 8, 99-106 (1979); Winter et al., Nature (London) 299, 756-758 (1982).

It is very well documented that the native human fibroblast interferon (IFN- $\beta$ ) gene has been cloned and expressed to high levels in E. coli, Taniguichi et al., Proc. Jpn. Acad. 55, 464-69 (1979);

Taniguichi et al., Gene 10, 11-15 (1980); Taniguichi et al., PNAS USA, 77, 5230-33 (1980); Goeddel et al., Nuc. Acids Res. 8, 4057-74 (1980); and Derynick et al. Nature (London) 287, 193-97 (1980). Further, Figure 1 of the instant application provides the DNA sequence of the native, human IFN- $\beta$ . The coding sequence for the native, human IFN- $\beta$  and the modified IFN- $\beta$  of the instant invention can be constructed by standard chemical oligonucleotide synthesis techniques in combination with ligation and cloning procedures, all available prior to October 19, 1982. (October 19, 1982 is the filing date of the great-grandparent application, U.S. Serial No. 435,154, now abandoned.)

European Patent Application 28,033 (published June, 1981) (Enclosure 7) states at page 19 that plasmid 319-13 has the fibroblast interferon mRNA sequence and said plasmid named TpIF 319-13 transformed into E. coli X1776 was deposited with the ATCC under accession number ATCC 31712. The base sequence of the mRNA sequence is given in Table 5 of that application (p. 18), which permitted prediction of the entire amino acid sequence for the native IFN- $\beta$ .

Therefore, as the active IFN- $\beta$  gene has been well-characterized, Applicants respectfully submit that preparing the source plasmid of Example 1, that is, p $\beta$ ltrp containing the IFN- $\beta$  gene would be well within the ordinary skill of the art. Plasmid p $\beta$ ltrp is derived from the well known pBR322 plasmid and contains the much used trp promoter 5' to the native IFN- $\beta$  gene. Many other possible vectors and promoters could be used as a vehicle to express the native IFN- $\beta$ , and anyone skilled in the art could easily make plasmids functionally comparable to p $\beta$ ltrp. Any such analogous vector carrying the native IFN- $\beta$  gene could be used to follow the experiments of Examples 1-11.

As shown in Example 1, a Hind III/Xho II DNA fragment from the plasmid p $\beta$ ltrp (Figure 3 provides a restriction map of this plasmid) contains the entire coding region of the native, human IFN- $\beta$  gene. This gene is simply inserted between the HindIII and BamHI restriction sites of the phage M13mp8 [See J. Messing, "Third Cleveland Symposium on Macromolecules: Recombinant DNA", Ed. A. Walton, Elsevier Press, 143-153 (1981)] replicative form (RF) DNA (See Figure 4). The phage DNA is then reacted with DNA polymerase I Klenow

fragment to convert it into double stranded (ds) DNA as shown in Figure 2. The T-A mutation, shown in Figure 2, results in the creation of a new HinfI restriction site in the IFN- $\beta$  gene.

The mutated clone is identified by using the oligonucleotide primer as a probe in a hybridization screening of the mutated phage plaques. The primer will have a single mismatch near the middle of the primer sequence when hybridized to the parent strain but will have a perfect match when hybridized to the mutated phage DNA. As described in great detail in Example 5, hybridization of the oligonucleotide primer is carried out whereby the primer preferentially hybridizes to the mutated DNA but not to the parent DNA. The newly generated HinfI site also serves as a means of confirming the single base mutation of the IFN- $\beta$  gene.

As described in Example 5, the M13 phage DNA carrying the mutated gene is isolated. Following isolation by routine techniques shown in Example 6, the mutated gene is spliced into an appropriate expression vector. As described in Example 6, dsDNA from the M13- $\beta$ 1 plaque having the mutated gene (M13-SY2501) was digested with restriction enzymes HindIII and XhoII and purified on a 1% agarose gel. The 520 bp insert fragment was inserted into a HindIII/BamHI digest of the expression vector identified as pTrp3, a plasmid derived from the well known plasmid pBR322 having the E. coli trp promoter inserted between the EcoRI and HindIII sites as shown in Figure 7. Obviously, any suitable expression vector could be used for this purpose.

Following ligation, the ligated DNA vector containing the mutated gene was transformed into E. coli strain MM294 (Meselson et al. (1968) Nature (London) 217, 1110-1113; MM-294 has been available to those skilled in the art (prior to October 19, 1982) from the E. coli Genetic Stock Center at Yale University). The transformed MM294 containing the expression vector having the mutated gene is identified in the specification as pSY2501. Figure 8a shows the HinfI restriction pattern of one of the pSY2501 clones comparing it with the HinfI pattern of the original IFN- $\beta$  clone, p $\beta$ 1trp. A restriction map

of clone pSY2501 is shown in Fig. 9 and the complete sequence is shown in Fig. 10 together with the predicted amino acid sequence.

Applicants respectfully submit that the plasmids and methods employed in the instant application are either well-known or capable of being prepared from documented sources by ordinary skill in the art. This application is not a case where a heretofore unknown or unidentified organism has been isolated, or where a new plasmid or host has been discovered or where a novel fusion partner of a hybridoma cell line is involved. This is a case where a known gene, native human IFN- $\beta$ , has been modified using materials, reagents, and techniques available and known to those skilled in the art at the time of filing the instant application.

Applicants request that if the Examiner disagrees with this assessment, that the Examiner point out with particularity which plasmids and microorganisms are considered to be "not known nor readily available to the skilled artisan."

Therefore, Applicants strenuously maintain that the specification with accompanying citations and references provide an enabling disclosure for one of ordinary skill in the art to make pSY2501 as well as analogous appropriately substituted plasmids. Applicants submit that it is well within the ordinary skill of the art to transform a host strain with pSY2501 or analogous plasmids and to express the desired IFN- $\beta$  position 17 mutein.

Applicants therefore respectfully submit that the deposits of the exemplary plasmid pSY2501 are more than sufficient to support the claims in view of the enabling disclosure.

#### References Made of Record

For the sake of completeness, Applicants are making of record the PTO form 1449 filed in the parent application (Enclosure 8).

Applicants also disclose to the Examiner their awareness that a three-way interference is now in progress in the U.S. Patent

and Trademark Office concerning U.S. applications presumably corresponding to Biogen's European patent application no. 41,313; JFJFCR's European application nos. 28,033 and 42,246; and Yeda Research's U.K. patent application 2,063,882. The Biogen and JFJFCR's applications were included in Form 1449, submitted in the parent application; a copy of the latter Yeda Research application is enclosed as Enclosure 9.

Applicants also make of record an article by Rastetter, Trends in Biotechnology, 1, pages 3-7 (1983) which discusses the concept of site-directed mutagenesis (Enclosure 10).

Applicants disclose as Enclosure 11 European patent application publication no. 128,467 which was published December 19, 1984 and has a U.S. priority date of June 1, 1983 (U.S. 499,964). That application is entitled, "Polypeptide Having Interferon Activity." Claim 1 reads:

1. A polypeptide having interferon activity characterized in that its amino acid sequence corresponds to the amino acid sequence of an interferon selected from a rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  wherein, however, at least one cysteine residue has been replaced by an amino acid residue which is incapable of forming an intermolecular disulfide bond.

Applicants, by making the instant references of record do not intend such act to be considered as an admission that any or all of the references constitute prior art or that any or all of the references, individually or in combination, render the instant claims unpatentable.

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Conclusion

Applicants respectfully submit that the above amendments and remarks render the instant claims in condition for allowance and earnestly request that the Examiner withdraw the §112 and §101 rejections and allow claims 44-47 and 49-54, all the claims of the instant application.

Respectfully submitted,  
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## New \$5-million lab gives Pharmacia base in Japan

**TAMATSUKURI, JAPAN**—"To get a foothold in Japan" is why Pharmacia AB (formerly Fortia AB) of Uppsala, Sweden, decided to begin building a \$5-million laboratory this month here, just outside Tsukuba, Japan's "science city," says the firm's vice president for diagnostic research, Jan Rosengren. The lab, to focus on diagnostics, pharmaceuticals, and product separation processes, "will look to pick up some new ideas from Japanese universities and industries, evaluate them in-house, then transfer any potential developmental work back to Sweden. We're making a long-term commitment to serve the Japanese market," Rosengren adds.

The new facility initially will employ about 10 scientists and technicians; when fully staffed, approximately 35. It will house a 250- to 400-square-meter chromatographic pilot plant, Rosengren says, "for demonstrations to the Japanese biotechnology industry."

Pharmacia, a medical sciences firm with production and research facilities in Denmark and the U.S., has a sales subsidiary in Tokyo to market its separation processes in this country. Green Cross, Ltd., of Osaka handles pharmaceuticals, while Shionogi & Co., Ltd., also of Osaka, and Daiichi Radioisotopes Labs of Tokyo distribute diagnostics.

## Cetus-Shell venture launches clinical trials of 2-cysteine interferon

**ROTTERDAM**—Using site-specific mutagenesis techniques, scientists at Cetus Corporation of Emeryville, Calif., have produced beta interferon in *Escherichia coli* with greater stability and about 10 times the specific activity than their earlier clones had shown. The feat was reported here in April to the International Congress on the Biology of Interferon by David Mark, manager of Cetus' lymphokines program.

Based on encouraging results from preliminary testing of their new genetically engineered interferon, the company applied to the U.S. Food and Drug Administration in mid-April for Investigational New Drug (IND) permission to test the product clinically. These human studies should begin in "a matter of weeks," says Jeffrey Price, Cetus vice president for research and development.

Prior animal and in vitro testing of the modified interferon indicates that it is very similar to the native molecule in its antiviral and immunological activity. It also appears to be nontoxic to animals.

Phase I trials for safety and dosage levels will be carried out by Drs. Ernest Borden at the Wisconsin Clinical Cancer Center in Madison and Thomas Merigan, a Cetus consultant at Stanford University School of Medicine. Then, after more dose-response tests, Phase II studies will measure the interferon's efficacy in treating a variety of conditions, says Dr. Behzad Khosrovi, Cetus director of development. They should start early in 1984, barring unexpected delays, and will take place at university hospitals around the U.S. Target diseases, Price tells *Newswatch*, will most likely include "viral infections such as hepatitis, herpes, and influenza and a broad spectrum of cancers, notably of the breast, colon, and prostate."

### Shell funding clinical trials

Funds for the planned clinical trials come from Cetus' joint venture with the Shell Oil Co. (*Newswatch*, April 18, p. 7). Price estimates that Cetus has spent about \$20 million in R&D money getting to this point—exclusive of construction of an interferon production plant. Shell, as joint-venture partner, will defray clinical-trial costs, which the first year should amount

to between one and two million dollars. Wisconsin's Dr. Borden is clinical director of the joint venture. Once FDA grants the new interferon final new-drug approval, Cetus fully intends to produce and market the product itself, says Price.

### Two cysteines beat three

As Mark explained to the congress here, the key to Cetus' creation of its beta-interferon variant lay in formulating and testing a hypothesis that there is a link between the molecule's disulfide chemistry and its degree of specific activity and stability. Human fibroblast (beta) interferon has three cysteine residues at key positions, only two of which are involved in forming an intramolecular disulfide bond. Thus the third cysteine residue can form covalent dimers with other interferon molecules, leading to a decrease in potency.

Mark decided to eliminate one of these amino acid residues by means of site-specific mutagenesis. His experiments hit upon the most available of the trio and changed it to serine, which has an oxygen atom where cysteine has a sulfur. He accomplished this switch by using an oligonucleotide containing a single base substitution in the cysteine codon, thereby changing the DNA code to serine.

This produced a beta-interferon molecule that, when purified, has a specific activity of  $1.3 \times 10^8$  international units/mg, which is comparable to the body's own interferon. The mutant form proved stable in storage for at least three months. In contrast, the interferon produced earlier by Cetus without this enhancing step had a specific activity of only  $3 \times 10^7$  units/mg and lost biological activity too rapidly for clinical use.

To protect what they see as a promising innovation, the firm filed a process patent application some time last year.

### Luck plus imagination did it

Across the bay in South San Francisco, Ron Wetzel, a senior scientist at Genentech, Inc., calls David Mark's result "quite exciting." It is, he says, "the first example of a practical application of site-specific mutagenesis used on a rational basis to make useful improvements in a protein's properties."

Wetzel adds that though many people have experimented with site-specific mutagenesis, Cetus' result represents the "first really significant practical accomplishment in this area." Asked why Mark and his team succeeded where others have not, he observes, "It was a bit of luck, imaginatively applied."

The fact that vials of Cetus' new interferon shows long-term shelf life is important. Hoffmann-La Roche has had to withdraw from clinical trials of its interferon because "there was a question of the stability of the product," says Roche spokesman John Doorley. And Robert Oldham, manager of the National Cancer Institute's Bioresponse Modifiers Program, cites a "falling off in antiviral activity" as the reason Roche backed off.

Cetus scientists believe their two-cysteine interferon has a good chance of avoiding such snags. Genentech's Wetzel is not convinced that the cysteine-serine switch is the only route to take for high yields and stability, but does see it as "a viable path so far."

## BRL, GIBCO to merge

GAITHERSBURG, MD.—The Dexter Corporation's GIBCO Division in Chagrin Falls, Ohio, will merge with Bethesda Research Laboratories, Inc. (BRL) here to form a firm with "combined revenues as big as any two of the 'Big Four' biotechnology R&D companies [Biogen, Cetus, Genentech, Genex] combined." So says the president of GIBCO, Charles D. Clark, who will be the new firm's chief executive officer. When will the merger take place? "Very rapidly, we hope," says Frederick R. Adler, managing partner of Adler and Company, a leading New York City and Palo Alto, Calif., venture-capital group and BRL's board chairman. Adler is the proposed chairman of the new firm. Once merged, the company will sell a portion of its stock through a public offering.

GIBCO sells mammalian and microbial culture media, parenteral pharmaceuticals, and vaccines for small mammals. Its net sales in 1982 were over \$77 million. BRL is a leading supplier of enzymes, reagents, and specialty products for biotechnology laboratories and is developing recombinant-DNA-based clinical diagnostics and therapeutics. Its 1982 sales were over \$13 million, up 30% from those of its troubled 1981 (*Newswatch*, March 15, '82, p. 1). "Together the two companies should have combined sales over \$100 million," Clark tells *Newswatch*.

"We'll make what can be sold today," says David L. Coffin, chairman of The Dexter Corp., Windsor Locks, Conn., which produces specialty materials and is the oldest company listed on the New York Stock Exchange. "And we'll use BRL's r-DNA expertise to develop viral diagnostics and therapeutics," he adds. "It's a merger made in heaven," says Adler.

Is that why Dexter's stock jumped 10 points in the two weeks following the April 28 announcement of the "planned" merger? "Only Wall Street knows for sure," Coffin says, but he adds wryly: "The new firm should have a chance of being profitable."

"We won't make any products like foot-and-mouth vaccine or human insulin," Clark says. "We'll leave that to the big pharmaceutical companies like Merck and Lilly." This runs counter to the latest strategy of Biogen, Cetus, and Genentech—all planning to do the clinical testing and marketing of their r-DNA-made pharmaceuticals and interferons themselves (*Newswatch*, March 7, p. 1).

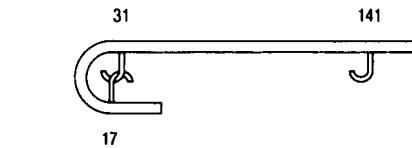
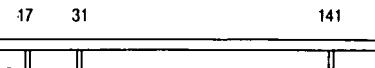
## \$61-million lab highlights Canada's bioprogram

MONTREAL—A two-year-old gleam in Canada's eye is about to become a \$61-million biotechnology R&D laboratory here, the cornerstone of a biotechnology strategy totaling about \$100 million Canadian (\$81 million U.S.) in federal investments. Construction will begin by the end of the year; completion is set for late 1985.

"Our efforts will be focused, at least initially, on animal diagnostics and vaccines, bacterial ore leaching,

## BETASERON™ (HUMAN RECOMBINANT BETA INTERFERON)

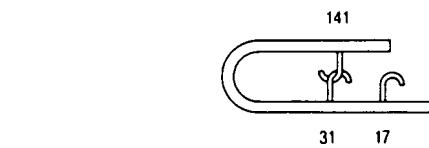
Cetus' advanced genetic engineering improved the product: In order to become active, recombinant interferon folds into a three dimensional shape by forming bonds between specific sites along the amino acid chain—positions 17, 31 and 141. Of the three bonds that can form, only one results in an active product. Since both the inactive products involve site 17, Cetus scientists modified the chain, eliminating site 17's bonding capability by substituting a serine for a cysteine amino acid at that site. The beta "ser" interferon they created, called BETASERON™ (human recombinant beta interferon), can form only the bond that results in an active and stable product.



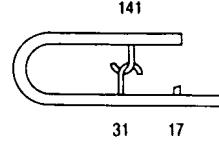
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## The human tumor colony-forming chemosensitivity assay: A biological and clinical review

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Key words: human tumor colony-forming assay, chemosensitivity assay

### Summary

Over forty papers describing correlations between *in vitro* human tumor sensitivity to a variety of chemotherapeutic agents and the *in vivo* response of patients to those agents have been published since the publication in 1978 by Salmon and Hamburger of their results of a human tumor colony-forming chemosensitivity assay (CFCA). The true positive rate in over 1600 correlations is 71% and the true negative rate is 94%. The biological elements of the assay, its developmental history, its place in the spectrum of *in vitro* chemosensitivity assays, and its theoretical and practical limitations are discussed. The scope, design, and limitations of key clinical trials are presented and an analysis of the potential errors of statistical interpretation of the trials as well as the results of the trials is given.

### Introduction

The choice of drugs in the systemic treatment of cancer is still, after more than three decades of chemotherapy, made empirically. Histology alone is an inadequate clue to the real responsiveness of an individual patient's tumor to a particular drug. Even in the most responsive tumors, such as testicular carcinoma or Hodgkin's lymphoma, there are still patients who will not respond to "first-line" regimens. Clinicians have about a 50 per cent chance of choosing effective drugs in the palliative treatment of tumors with an intermediate degree of responsiveness, such as breast or ovarian cancer, and must face the dilemma of deciding whether or not to offer chemotherapy at all to the very poorly responsive tumor types such as non-small cell lung

or colon cancer. The difficult decision of choosing initial therapy is easier than the choice of secondary therapy after initial non-response or relapse. Because of the limited accuracy of drug choice based upon clinical criteria alone, the utility of a test by which the sensitivity of an individual patient's tumor could be determined is obvious. Many efforts to develop such a test have been made. This article reviews the use of tumor colony growth in an *in vitro* agar system, the human tumor colony-forming chemosensitivity assay (CFCA) as the basis for such a test, describes how it fits into the spectrum of three decades of such *in vitro* assays, discusses technical, biological, and statistical limitations of this assay, and presents a comprehensive literature review of the results of clinical trials using this assay.

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### Biological basis for the human tumor colony-forming chemosensitivity assay (CFCA)

The growth of malignant cells in a soft-agar medium is not new. In fact, anchorage independent cell proliferation in agar has long been one of the characteristics which has defined the transformed state (1). While many laboratories have attempted the long-term culture *in vitro* of human tumors, the difficulty of doing so has resulted in most experimentation being confined to cell lines capable of long term passage *in vitro* and reproducible growth in an agar system. With the development of more supportive cell culture media and the increased interest in developing cell lines from spontaneous human tumors, the last decade has witnessed the growth of many tumor cell line "banks" which now contain hundreds of unique, well characterized lines of primary human tumors. However, because of the low success rate in growing fresh, non-passaged spontaneous human tumors most laboratories directed their efforts to chemosensitivity systems using established cell lines.

In the mid 1970's Hamburger and Salmon applied advances in cell culture to the growth of primary human tumors in agar (2). After developing a growth-permissive media, they demonstrated that a large percentage of myeloma specimens could form colonies of plasma cells in agar. They then expanded this work to ovarian carcinoma. Tumor specimens, either from solid tumor masses or malignant ascites, were mechanically disaggregated into a suspension, which was as close to a "single-cell suspension" as possible. These cells, which included both tumor cells and stromal cells of variable viability were suspended in an agar-containing culture medium and then layered or "plated" onto a semi-solid underlayer. The underlayer prevented normal human fibroblasts, a major tumor stromal component, from adhering to the culture dish bottom and forming colonies which might be confused with colonies of tumor-cell origin. After 7 to 14 days of incubation in a humid, CO<sub>2</sub>-enriched atmosphere at 37°C, colonies, defined as new, round aggregates of 30-50 tumor cells, could be seen. A variety of morphologic (3, 4), chromosomal (5), biologic, and immunologic

(6, 7) studies have demonstrated that almost all of the colonies are consistent with a tumor-cell origin. Because of the inability to assure colony formation in all cases from single cells, the term "clonogenic" which was often applied to the assay is in a strict sense inaccurate (8). While the "plating efficiency" i.e., the number of colonies formed from a given number of cells plated was low, (0.1% to 0.001%) Salmon and Hamburger could demonstrate colony formation in over half of all tumor specimens plated.

Hamburger and Salmon then studied the effect of commonly used cytotoxic drugs on the growth of these tumor colonies. Tumor cells were incubated with the cytotoxic drug to be tested for one hour. Drug concentrations were based upon the serum levels obtained after clinical administration. The most commonly used concentration was one tenth of the peak serum concentration obtained after a standard dose. All cells were then washed and plated. After 10-14 days, colony formation by drug-exposed cells was compared to colony formation by an aliquot of control cells which were not exposed to drug. In 1978 they published a comparison of the degree of inhibition of colony formation of a particular patient's tumor cells exposed to a particular drug to the actual *in vivo* response of the same patient to that drug. Their results in this retrospective, correlative trial showed a statistically significant correlation between the inhibition of colony formation *in vitro* and the patient response *in vivo* (9). The publication of this very exciting work in a journal read widely by clinicians (and by the lay media) generated renewed enthusiasm in the development of an *in vitro* predictive assay. Many laboratories undertook similar work in an attempt to corroborate and further expand the applicability of this colony-forming chemosensitivity assay. In the five years since the publication of Hamburger and Salmon's initial clinical observations, over thirty laboratories using similar techniques have published results of correlations between *in vitro* and *in vivo* responses to a great variety of drugs. These correlations, and the biological relevance and limitations of the assay upon which they are based, are discussed below.

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### The position of the colony-forming chemosensitivity assay in the spectrum of chemosensitivity assays

While an exhaustive review of all of the biological bases for predictive chemosensitivity assays which have been investigated is beyond the scope of this review, it is worthwhile to point out where in the spectrum of such assays the CFCA fits.

Most assays can be put comfortably into one of five types. The first, a pharmacokinetic-presumptive model, assumes that drug uptake or the presence of drug receptors is predictive for a cellular response (10). While this is often true, there are serious limitations to this approach. First, while drug uptake may be necessary for drug activity, it may not be sufficient. Secondly, the ability to measure intracellular drug concentrations or the presence of a receptor is limited to a small number of drugs and cell lines. Assay conditions may alter membrane transport function from those active *in vivo*. Finally, recent work by Tritton suggests that some drugs, such as doxorubicin, may not require entry into the cell to influence proliferation (11). A second model looks at morphologic evidence for damage of the tumor cell following drug exposure. This straight-forward approach is employed in many cytotoxicity screening assays but has many limitations. First, in a heterogeneous population of tumor cells and stromal cells in a tumor specimen, it is difficult to accurately identify only the tumor cells. Secondly, it is impossible to separate out tumor cells with little or no proliferative potential from cells which will behave in an aggressive fashion *in vivo*. Third, many cells which, after drug exposure, will appear to be perfectly normal have in fact sustained a loss of reproductive ability, and many cells which appear morphologically damaged may go on to recovery. In addition a morphologic approach is difficult to standardize and has much inter-observer variation. A third model looks for evidence of altered or impaired cellular metabolism or nucleotide synthesis. Numerous indices have been evaluated in this category: changes in cellular glycolysis, activity of cellular dehydrogenases, incorporation of labelled thymidine, or other amino acids, rates of DNA or RNA synthesis, and so forth

(10). While more biochemically complex than many of the other tests, these assays still suffer from an inability to identify the appropriate cell population (tumor cells as opposed to tumor stroma) and from cells which have proliferative, tumor-generating, capacity (the so-called "stem cells") from cells which are unable to proliferate to a biologically relevant degree. And, as in the case with morphologic assays, many cells with impaired functional indices would recover and many cells which showed little evidence of functional impairment would have never been able to proliferate. A fourth approach has been to assess loss of cell viability after drug exposure either by looking for release of radio-labelled chromium, by the exclusion of a vital dye such as trypan blue, or by the loss of adherence in culture. The fifth model, of which the CFCA is an example, is one in which proliferative capacity of the drug-exposed tumor cells is measured as an end-point. This parallels the *in vivo* setting where tumor growth is the relevant end-point for drug activity. It also deals with the question of proliferative heterogeneity within tumor populations. As performed, however, in the CFCA, it has many theoretical and practical drawbacks and limitations in its application to clinical chemosensitivity determination for an individual patient. These limitations will be discussed in the next section. For a more detailed discussion of the biological elements of chemosensitivity assays, the reader is referred to two excellent comparative reviews which have been recently published (10, 12).

### Theoretical and practical problems of the colony forming chemosensitivity assay

A variety of factors, both practical and theoretical, make performance and interpretation of the CFCA difficult. The most immediate and obvious drawback is the limited accessibility of tumor tissue. Malignant pleural effusions and ascites offer the most available and easily obtained sources of cells, but the majority of patients with solid tumors have tumor specimens available only at time of initial diagnostic or therapeutic surgical resection. Coordination and cooperation between surgeon, pathol-

ogy department, and testing laboratory is necessary to allow the performance of the assay. Once a tumor specimen is obtained, it is often difficult, if not impossible, to obtain an acceptable cell dispersion. Few tumor preparations are truly "single cell". Occasionally there are so many large clumps of tumor cells that determination of colony growth is made difficult by the task of discriminating between new colonies formed from single cells and colonies which are really just clumps of cells which have remained viable or have gone through a minimal number of divisions during the incubation period. Automated counting systems which compare day 1 to day 14 counts of cellular aggregates can, in part, compensate for this difficulty but the preparation of a suitable cellular suspension remains a formidable problem in most laboratories. In addition, the definition of a colony is arbitrary. Is an aggregate of only 30 cells large enough? Is an aggregate of 50 cells too demanding a criterion for growth? Can a fixed size for a colony be applied to tumors with very small cells and ones with very large cells equally? Since the biological basis for the assay is the exposure of dividing cells to a drug, the critical issue is the number of cell divisions which each cell has gone through on its way to becoming a colony. If all colonies arose from single cells, of uniform size, colony size could correlate with the number of cell divisions. In many specimens, however, some colonies arise from single cells but others arise from small clusters of cells. The term "colony" depends upon functional definitions which need to be standardized within each laboratory and for each tumor type.

The *in vitro* pharmacology of routinely used drugs is largely unknown. A few drugs, such as cyclophosphamide, are activated *in vivo* by hepatic metabolism and are inactive *in vitro*. Some drugs are cell cycle specific; some are more cytotoxic in dividing cells, and some appear to work independently of cell cycle. The exposure of the cells to drug for one hour is arbitrary and far from the actual clinical exposure time in the case of many of the drugs used. Many laboratories test only one drug concentration. In addition to not demonstrating a dose-response curve, which would give greater assurance that the inhibition of colony for-

mation being seen had the desired biological relevance and was not an *in vitro* artifact of non-specific cytotoxicity, the use of only one dose may not parallel the concentration to which the tumor cell is exposed *in vivo*. For drugs and tumors in which an *in vivo* dose-response relationship exists, the establishment of an *in vitro* dose-response curve is important.

The determination of the degree of inhibition of colony formation necessary to call a tumor "sensitive" is arbitrary. While most authors have used some fixed percentage of inhibition of colony formation compared to control, i.e. 50% inhibition or 70% inhibition, and have stated that inhibition of less than that amount meant "resistance" and more than that indicated "sensitivity", there is in fact a continuum in the relationship between colony inhibition and clinical response (13). The greater the inhibition of colony formation, the greater the likelihood of clinical response, and there is no finite cut off point to mark "resistance" or "sensitivity".

Another disadvantage is that the assay cannot be repeated because of the unavailability of tumor cells. Several investigators have frozen aliquots of tumor specimens and have performed chemosensitivity assays at a later date with similar chemosensitivity results but lower plating efficiency compared to the initial assay. (D.D. Von Hoff, personal communication). Usually, however, there are not adequate numbers of tumor cells from one specimen to make this a routine procedure. The inability, therefore, to repeat the assay is not satisfactory from both a scientific point of view, where experimental data should be verifiable, and as a practical matter in the event of an unsuccessful assay.

Another major practical difficulty is the small percentage of tumors in which there is adequate *in vitro* growth for an evaluable chemosensitivity assay. The percentage of specimens which result in an evaluable chemosensitivity assay vary from 20% to 90% depending upon the laboratory, the tumor type being assayed, and the laboratory's definition of evalability. In one series published by Von Hoff, 31% of over 800 specimens received ultimately yielded an evaluable assay (14). In a large trial sponsored by the National Cancer Institute comparing the results of four different labora-

tories, only 25% received (inadequate growth) evalability (15). Failure of the technique is the successful and currently possible tool of the assay being tested. Most epithelial tumors in fact resist better opportunity predictive ability.

Table 1. Forty re-

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Banerjee, T.K.
Bernheim, J.L.
Bogden, A.E.
Carney, D.
Durie, B.G.

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tories, only 25% of all tumor specimens provided adequate growth and satisfied strict criteria for evaluable (15). While many of the reasons for failure of the drug assay lie with the specimen received (inadequate numbers of tumor cells, contaminated specimens, and so forth) and not with the technique of the assay itself, it is still true that the successful assay of all human tumors is not currently possible. The final shortcoming as a clinical tool of the assay is the ineffectiveness of the drugs being tested. Most assays predict resistance because most epithelial cancers are, at the time of the assay in fact resistant. New, effective drugs will give a better opportunity for the assay to demonstrate its predictive ability.

### The results of clinical trials of the *in vitro* colony-forming assay

In spite of the fact that only 25-50% of all specimens eventually result in an evaluable assay, almost two thousand correlations have now been reported between *in vitro* and *in vivo* responses to conventional and investigational drugs. This paper summarizes forty published reports of these *in vitro*-*in vivo* correlations (Table 1). Each trial falls into one of three basic designs. The first, and most common, is a retrospective, correlative study. In this study design a tumor specimen is obtained from a patient who has been treated with one or more drugs, as a single agent or in combination. Results

Table 1. Forty reports on *in vitro*-*in vivo* correlations.

Author	# of Pts.	# of in vitro- <i>in vivo</i> correlations	Trial design	Clinical results				Tumor types and clinical comments	Ref.
				+ / +	+ / -	- / +	- / -		
Ahmann, F.R.	8	8	Prospective	3 + / +			5 - / -	Solid tumors, prior all pts treated with AMSA	23
Alberts, D.S.	40	95	Prospective	13 + / +	8 + / -	1 - / +	73 - / -	Previously treated, relapsed, ovarian carcinoma	24
Alberts, D.S.	6	6	Decision-aiding	5 + / +			1 - / -	Relapsed solid tumors treated with bisantrene	22
Alberts, D.S.	54	54	Decision-aiding	assay directed survival = 10.5 mos. clinician directed survival = 3.0 mos.				Relapsed, ovarian carcinoma	19
Ballet	5	5	Prospective	2 + / +			3 - / -	Untreated effusions, all carcinomas	25
Banerjee, T.K.	47	53	Retrospective	19 + / +	9 + / -	3 - / +	22 - / -	GI, GU, breast, sarcomas, lung some treated	26
Bernheim, J.L.	11	32	Retrospective	8 + / +	1 + / -		23 - / -	Lung, breast, GI ovarian, CTC, some treated	27
Bogden, A.E.	10	10	Correlative				10 - / -	Solid tumors. to sub-renal capsular assay	28
Carney, D.	26	147	Prospective and retrospective Correlative	49 + / +	9 + / -		89 - / -	Small cell bronchogenic some pre-treated	18
Durie, B.G.	13	17	Prospective	12 + / +	4 + / -		1 - / -	Leukemia, majority in relapse	29

Table 1. Continued.

Author	# of Pts.	# of in vitro-in vivo correlations	Trial design	Clinical results				Tumor types and clinical comments	Ref.	Author	
				+/+	+/−	−/+	−/−				
Epstein, L.B.	8	12	Retrospective	7+/+				5−/−	Ovarian carcinoma	30	Rey, A.
Gau, T.C.	9	11	Correlative					5−/−	Untreated colon CA with FUDR and Mito-C	17	Riou, G.
Hogan, W.M.	41	67	Retrospective and prospective	7+/+	9+/−	4−/+	47−/−	Ovarian, many pre-treated		31	Rosenblum, M.
Kaiser, L.R.		12	Correlative	3+/+	1+/−			8−/−	Bronchogenic carcinoma	31	Rupniak, T.H.
Kern, D.H.	84	84	Prospective	21+/+	4+/−	5−/+	54−/−	Melanoma, lung, colon, gastric, breast, some treated		32	Salmon, S.E.
Ludwig, H.	14	21	Decision-aiding	21+/+	3+/−			4−/−	Multiple myeloma 11 of 14 patients had clinical response based upon chemotherapy chosen by assay results	21	Simmonds, A.
Mann, B.D.	32	37	Retrospective	9+/+	2+/−	1−/+	26−/−	Melanoma, gastric sarcoma, lung many relapsed		33	Stanisic, T.H.
Mann, B.D.	11	11	Prospective	4+/+	1+/−			6−/−	Melanoma treated with DTIC + hyperthermia	34	Tisman, G.
Meyskens, F.L.	34	45	Decision-aiding	7+/+	8+/−	4−/+	26−/−	Melanoma		20	Tveit, K.M.
Meyskens, F.L.	24	37	Retrospective and prospective	12+/+	7+/−	4−/+	23−/−	Melanoma, majority pre-treated and relapsed		35	Von Hoff, D.D.
Natale, R.B.	13	15	Correlative	3+/+	2+/−	1−/+	9−/−	Transitional cell bladder cancer		36	
Natale, R.B.	28	28	Prospective	10+/+	3+/−	1−/+	14−/−	Ovarian carcinoma		37	
Paganuzzi, M.	14	35	Correlative	“83% true+, 82% true−”				Pediatric malignancies		38	
Park, C.H.		34	Prospective	13+/+	5+/−	1−/+	15−/−	ANLL, some patients previously treated		39	
Parker, R.L.	6	6	Correlative	2+/+	1+/−	0−/+	3−/−	Cervical carcinoma		40	
Plasse, T.	16	16	Correlative	7+/+	1+/−	1−/+	7−/−	Breast, colon, lung, melanoma, ovary		41	
Possinger, K.	94	94	Correlative	24+/+	13+/−			Breast, ovarian, lung		53	Wilson, A.P.

Table 1. Continued

s and ments	Ref.	Author	# of Pts.	# of in vitro- in vivo correlations	Trial design	Clinical results				Tumor types and clinical comments	Ref.
						+/+	+-	-/+	-/-		
	30	Rey, A.	7	7	Correlative	2+/+			5-/-	Breast, ovarian, glioblastoma, meningioma	42
colon	17	Riou, G.	40	95	Correlative	"86 of 95 (91%) accurate"				Ovarian carci- noma	43
UDR		Rosenblum, M.L.	15	15	Prospective	3+/+	4+/-		8-/-	Glioblastomas treated with BCNU	44
C	31	Rupniak, T.H.	2	4	Correlative				4-/-	Courtney tech- nique, ovarian carcinoma	45
nic car-	31	Salmon, S.E.	153	250	Retrospective and prospect- ive	56+/+	23+/-	7-/+	166-/-	Ovarian, mye- loma, many carci- nomas, many pre- viously treated	46
lung,	32	Simmonds, A.P.	7	7	Correlative	"6 of 7 correct correlation"				Breast, gastric, osteosarcoma	47
ic,		Stanisic, T.H.	5	9	Prospective	"7 of 9 accurate" (78%)				Bladder cancer treated with Mito- C thio-tepa	48
othera- by as-		Tihon, C.	21	21	Retrospective	6+/+	1+/-	1-/+	13-/-	Breast, lung, colon; ovarian many previously treated	49
	33	Tisman, G.	68	120	Retrospective and prospect- ive	13+/+	12+/-	1-/+	98-/-	Mostly carci- nomas, some pre- treated	50
treated	34	Tveit, K.M.	39	49	Correlative	10+/+	1+/-		42-/-	Courtenay method, all melano- mas	51
C + mia		Von Hoff, D.D.	101	123	Retrospective	15+/+	6+/-	2-/+	100-/-	Thirty six histol- ogic types; lung, breast, head and neck, ovarian, many pre-treated	14
a, major-	20									Two major and two minor re- sponses in pa- tients treated	
a, major- ated	35										
nal cell	36	Von Hoff, D.D.	246	246	Decision-aiding	26+/+	17+/-	31-/+	100-/-	Pre-treated colon, lung, ovarian, melanoma, breast.	13
cancer										Assay-directed re- sponse rate = 25%, clinician directed response = 11%	
carci-	40	Wilson, A.P.	8	8	Correlative	"correct correlation in 8 patients receiving first line chemotherapy"				Ovarian	52
colon,	41										
anoma,											
ovarian,	53										

of the assay are compared to the reported clinical response of the patient to a tested drug. A prospective correlative trial is similar except that the tumor specimen is tested before the patient is treated. In both correlative trial designs the laboratory performing the *in vitro* assay and clinicians treating the patient are unaware of each other's observations. The correlative design used in these studies is identical to the design used for the majority of "predictive" tests used in medicine, such as the determination of the amount of estrogen receptor protein in breast cancer tissue in order to "predict" response to hormonal therapy. The third study design, the prospective "decision-aiding" design, is the most rigorous way of demonstrating clinical usefulness of a test. In this design the outcome of therapy based upon the results of the assay are compared to the therapeutic outcome using standard clinical judgement based upon information relevant to an individual patient's case.

To summarize the results of all of the trials presented in Table 1, 1667 comparisons of *in vitro* and *in vivo* responses have been reported in over 1300 patients. The tumor types studied include responsive tumor types (myeloma, testicular carcinoma) but the majority are of tumor types either initially refractory to chemotherapy (colon, lung) or tumors which have relapsed following initial chemotherapy (breast, ovarian). Table 2 summarizes the correlations. Defining the percentage of "true positives" as

true positives

(true positives & false positives)

and the percentage of true negatives as

Table 2. *In vitro*-*in vivo* correlations of drug sensitivity.

Human tumor colony-forming assay results		
	Sensitive(+)      Resistant(-)	
Clinical response(+)	375 +/+	64 -/+
No clinical response(-)	156 +/-	1072 -/-

true negatives

(true negatives & false negatives)

the overall true positive percentage is 71% and the true negative is 94%. The sensitivity of the test, defined as

true positive

(true positive & false negative)

is 85%, and the specificity, defined as

true negative

(false positive & true negative)

is 88%.

While analysis of the results of these studies in this way gives a satisfying number which implies a reliable predictiveness based, as it is, on a large and presumably statistically secure data base, several problems need to be recognized before an honest interpretation of the results of the studies can be made. First, *in vitro* results are for a single drug tested against the tumor target. Many studies have counted a corresponding clinical response to have occurred if a patient responded to a combination of drugs which included the tested drug. While many of the *in vitro*-*in vivo* correlations are with one drug, some are with combinations, and many authors do not state specifically how the issue of patient treatment with drug combinations was handled. Very little work studying the effect *in vitro* of the combination of drugs, or the correlation of combinations *in vitro* and also *in vivo* has been done. A second, and more serious drawback is related to the underlying probability that a particular clinical event will occur. For example, the overall true positive rate seen in the studies reported above was 72%. While this is excellent for diseases in which a response is unlikely, such as colon carcinoma, it is not much better than a clinically educated guess in diseases like myeloma, testicular cancer, or small cell carcinoma of the lung where a high initial response rate is expected. Similarly, a true negative percentage of 94% adds very little to the clinical estimation of the likelihood of response in a patient with colon cancer or melanoma. It is worthwhile to look at several large correlative series in which the patient treatment and tumor character-

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istics are well described. Meyskens *et al.* (16) reported a series of 24 patients with metastatic melanoma, many of whom had been previously treated. Since the clinical likelihood of a response in that population is small, the reported true negative rate of 86% is no better than an educated guess by the clinician. The true positive rate of 63%, however, is much better than a guess based upon knowledge of the drug used and the usual clinical response of melanoma. In a series of patients reported by Gau *et al.* (17) of patients with colon cancer metastatic to liver and treated with continuous infusion of FUDR, the surprisingly high *in vitro* response rate correlated well with the high clinical response rate seen. And similarly, in a series of patients with small cell bronchogenic carcinoma reported by Carney *et al.* (18) a large number of whom would be expected to respond, the true positive rate of 84% is not surprising but the true negative rate of 100% is impressive. Overall, while the patient populations vary markedly from study to study in terms of tumor type, patient heterogeneity, and presence or absence of prior therapy, the results — a true positive rate of 71% and a true negative rate of 94% — are seen in almost all of the reports in spite of the heterogeneity of patient characteristics and the variable likelihood of clinical tumor response. While not proving it, the data nevertheless supports the conclusion that it is the accuracy of the assay, not the underlying likelihood of clinical response given the patient population, that is responsible for the observed correlation.

A much more demanding study design, however, is that in which a clinical treatment decision is based upon the outcome of the test and then the outcome of the assay directed therapy is compared to therapy directed by a clinical judgement. The perfect "decision-aiding" trial would be one in which patients, after having a chemosensitivity test performed and a drug selected by the assay, would then be randomized to either receive the drug predicted by the assay to be active or the physician-chosen drug appropriate to the clinical circumstances. A number of practical considerations make this sort of trial difficult to do. Only one-third of all patients who have specimens submitted for chemosensitivity assay will have enough colony growth *in vitro* to

evaluate. Secondly, because of the inherent resistance of most solid tumors to chemotherapy, only a minority of tumors which can be evaluated *in vitro* would show sensitivity to a particular drug.

And finally, how does one handle the fact that in many cases the clinician would choose the same drug which the assay indicated to be active? Several prospective, decision-aiding trials have been performed. The largest, by Von Hoff *et al.* (13), looked at over 200 patients who were treated in the following fashion. If the assay resulted in successful tumor colony growth, the patient was treated with the drug which caused the greatest amount of colony inhibition, even if it was not the 70% inhibition which most investigators have defined as the lower limit for drug activity. If the assay was technically inadequate the patient would be treated by the clinician's choice. The highest clinical response rate (25%) was achieved in the patients, the majority of whom had solid tumors refractory to prior chemotherapy, whose chemotherapy was chosen based upon the assay results. A 15% response rate was achieved in patients for whom the assay could not be evaluated because of inadequate tumor growth and who were treated with the drug of their physician's choice. In the 102 cases where the assay did suggest a drug to which the tumor showed sensitivity but the clinician chose for clinical reasons not to use the assay-indicated drug, there was only an 11% response rate. The differences between assay-directed therapy and clinician-directed therapy would have been even greater if standard criteria for *in vitro* response had been used. Another prospective, decision-aiding trial was performed by Alberts *et al.* (19) in patients with ovarian carcinoma who had relapsed following standard chemotherapy. Of the 54 patients with a successful assay, 17 showed resistance to all drugs tested, and 37 showed sensitivity to at least one drug. In approximately half the patients in whom there had been an active drug based on the assay, clinical therapy was chosen based on the *in vitro* results. In the other half the clinician empirically chose the therapy. Survival from the initiation of therapy in the assay-directed patients was 10.5 months; survival in the patients treated empirically was 3.0 months. This difference was statistically significant

( $p = .005$ ). After two years of observation and continued patient accrual since the publication of these results Alberts has continued to see a significantly prolonged survival in patients who receive assay-directed therapy. It appears that *in vitro* sensitivity is not merely a biological marker for a more favorable sub-group of patients, for in this study patients who were sensitive to a drug *in vitro* but were not treated with the assay-directed drug showed the same survival as those patients who were resistant to all drugs tested and treated empirically. (Alberts, personal communication). Meyskens treated thirty-four patients with melanoma with the drug which showed most activity in the CFCA and observed a clinical response rate of 47%. This was significantly better ( $p = .02$ ) than the response rate of 20% seen in the previous 86 patients treated in the same institution on the basis of a clinical choice (20). Ludwig treated 14 patients with myeloma with drugs identified as active by the CFCA and achieved a clinical response in 11, for a response rate of 80% (21). Alberts treated six patients with solid tumors which were refractory to conventional chemotherapy and who were sensitive in the CFCA to bisantrene with that experimental drug. Of the six, five had a clinical response, a much higher percentage than one would expect with a phase II drug in refractory tumors (22).

None of these decision-aiding trials are perfect in their design. All can be criticized because of the many potential sources of physician bias as a result of the lack of strict randomization. All the trials do support, however, the hypothesis that the CFCA correctly identifies drugs to which an individual patient's tumor will be sensitive *in vivo*.

## Conclusion

Very little of the clinical practice of medicine is based upon the results of relevant, adequately controlled, and widely reproduced clinical trials. This is still true for the selection of drug therapy for patients with cancer. The results of the trials discussed above support the initial observations of Salmon and Hamburger. Even more encouraging is the positive trend seen in the results of the decision-

aiding trials. The technical limitations of a colony forming assay: poor growth, colony artifact, possibly inappropriate *in vitro* drug incubation conditions, and arbitrary cut-offs for sensitivity or resistance, are being improved with better disaggregation techniques, more appropriate media, *in vitro* bio-activation of drugs, and a better understanding of the relevant pharmacology of the drugs being studied and its relationship to the *in vivo* situation. It is naive to think that all tumors will grow best under one set of *in vitro* conditions and that all drugs should be tested in the same way. The development of more appropriate conditions should result in an improvement in correlative accuracy. In addition, as a larger number of assays in a particular tumor type against a single drug or a fixed combination are performed, better and more valid comparisons can be made. This, hopefully, will allow a decrease in the "apples and oranges" data aggregations which now comprise the bulk of the correlative literature. Future assays may well be tailored to the tumor type or particular drug being evaluated. Improvements in growth rate and thereby the percentage of evaluable tumor specimens will facilitate the completion of more randomized, decision-aiding trials which will be necessary to confirm the clinical usefulness of this assay.

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fluenza virus (Finter, 1967a, 1968), and replacing medium with suspensions of erythrocytes which adsorb to infected cells. After washing monolayers, red cells are lysed by hypotonic solution and liberated haemoglobin is quantitated by optical density measurement. A modification of this method has been described using  $^{51}\text{Cr}$ -labelled erythrocytes to quantitate haemadsorption (Emodi *et al.*, 1975a).

#### d) Reduction of Neuraminidase Yields

Reduction of the neuraminidase produced by influenza virus has been used to assay chicken interferon (Sedmak and Grossberg, 1973), human interferons, monkey, rabbit, hamster and mouse interferons (Sedmak, Grossberg, and Jameson, 1975). This method is reported to be reproducible, rapid, sensitive and convenient, provided the cell system used is a good producer of neuraminidase.

### 3. Cytopathic Effect (CPE)-Inhibition Assays

A number of viruses cause cell damage that can be visualized in the light microscope, and any of these cytopathic effects can be used to quantitate protection of cells by interferon. Two major methods of this assay are in wide usage, one based on microscopic reading of cell damage and one based on an indirect measure of the cell damage by amounts of a vital dye taken up by cells.

#### a) CPE-Reading Method

This method was first used by Ho and Enders (1959a), and has been used to assay nearly every type of interferon that has been described, against a great number of viruses (Sellers and Fitzpatrick, 1962; Fantes, O'Neill and Mason, 1964; Wheelock and Sibley, 1965; Kono and Ho, 1965; Bucknall, 1967; Billiau and Buckler, 1970; Bucknall, 1970; Ahl and Rump, 1976; Viehauser, 1977). The main advantages of this method are simplicity, speed and economy of samples and supplies, with introduction of semimicro-titration trays (Tilles and Finland, 1968; Dahl and Degre, 1972; Dahl, 1973).

I shall describe the semimicroassay system used in this laboratory that allows two workers to assay thousands of samples of human and murine interferons each week with nearly 100% success-rate.

Growth medium is introduced into each well of 96 well plastic microtiter trays, 50  $\mu\text{l}$ /well. The interferon sample (25  $\mu\text{l}$ ) is introduced into the first well of the row and the solution is mixed with a fresh micropipette tip. An aliquot (25  $\mu\text{l}$ ) is transferred to the next well and mixed with a fresh micropipette tip<sup>2</sup>, etc. After dilutions are made, including on each tray a standardized interferon preparation assay series and wells for virus controls and cell controls, trays are exposed to sterilizing ultraviolet-irradiation (Stewart II and Sulkin, 1966). Freshly trypsinized cell suspensions are then introduced into each well (0.1 ml containing about  $2 \times 10^4$  cells) and trays are incubated at 37° C overnight. A

<sup>2</sup> If serial dilutions are made with the same tip throughout, artificially high titers can be produced (T. Chudzio, 1977, personal communication).

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suspension of vesicular stomatitis virus containing about  $10^4$  plaque-forming units in 50  $\mu$ l of serum-free medium is introduced into each well, except cell controls, and trays are incubated about 24 hours at 37° C, at which time virus controls show 100% CPE. Endpoints are read as 50% protection, and as illustrated in Figure 3, are reliable within 0.5  $\log_{10}$  dilutions and end-point can be interpolated reproducibly to 0.3  $\log_{10}$  differences. However, at this level, the least satisfactory aspect of this assay method imposes itself, for subjectivity must decide differences of less than 2-fold.

b) Dye-Uptake Method

This modification of the CPE-inhibition assay, introduced by Finter (1969), relieves the imagination of the worker from visually reading the CPE. When the virus-induced damage has developed, a vital dye, neutral red (about 10<sup>-5</sup> gm %), is added to all cultures which are incubated for 2 hours. Cultures are then washed and dye which was taken up by living cells is eluted into acid-alcohol and quantitated colorimetrically. This method requires considerably more manipulations than the CPE-reading method but has the same sensitivity and is more precise (Finter, 1969; McLaren, 1970).

This dye-binding method for rabbit is currently in use 1972; McManus, 19

As it is possible precursors into the virus inhibition by Giron (1970) assayed media of MM virus several hours cells was precipitated with The endpoint was (INAS<sub>50</sub>) units. This results (McWilliam satisfactorily in a Kohler, and Wyler, tion of this method (Suzuki, Akaboshi, a

A similar method determining the amount double-stranded RNA (cells with Actinomyce

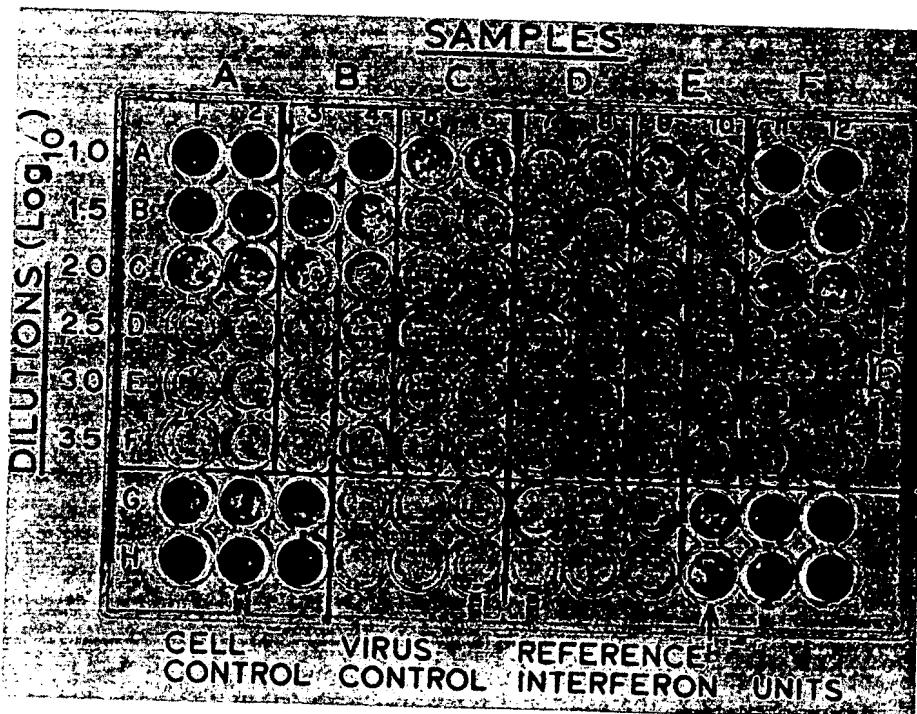


Fig. 3. Microtiter tray assay by CPE-reading method. The titers of the samples estimated from visual microscopic readings were: A, 100 units/ml; B, 60 units/ml; C, 20 units/ml; D, <10 units/ml; E, ~10 units/ml; F, 200 units/ml. Reference interferon assay 1 unit at arrow

Numerous other developed and most o utilities.

a) The ability of inter be measured in term feron-treated cells. The reverse-transcriptase-r Salzberg, 1976). Cul cells infected with M with interferon and i was then assayed for thymidine). This meth the PDD<sub>50</sub>-VSV meth ter, reproducibility an 24 hour period. Presu interferons.

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Table 18. Clinical trials with interferons

Disease	Treatment <sup>a</sup>	Effect <sup>b</sup> demonstrated	References
<b>A. Antiviral studies</b>			
<b>1. Localized infections</b>			
<i>a) Prophylaxis</i>			
Vaccinia	~200 unit (monkey) (id)	+	Scientific Committee on Interferon (1962)
Vaccinia	5 × 10 <sup>4</sup> units (human leukocyte or fibroblast) (id)	+	Scott et al. (1977 b, c)
Rhinovirus, parainfluenza-1, coxsackie A-21	~10 <sup>4</sup> units (monkey) (in)	-	Scientific Committee on Interferon (1965)
Rhinovirus	10 <sup>5</sup> units (human leukocyte) (in)	-	Tyrrell and Reed (1973)
Rhinovirus	10 <sup>7</sup> units (human leukocyte) (in)	+	Merigan et al. (1973 a)
Influenza	~10 <sup>4</sup> to 10 <sup>5</sup> units (human leukocyte) (in)	+	Soloviev (1967, 1969)
Influenza	10 <sup>6</sup> units (human leukocyte) (in)	±	Merigan et al. (1973 a)
<i>b) Therapy</i>			
Vaccinia gangrenosa	? (monkey) (sc)	-	Connolly, Dick, and Field (1962)
Post-vaccinal skin lesions	c 4000 units/gm (ointment) (human leukocyte)	+	Soos et al. (1972)
Vaccinia keratitis	? (monkey) (eyedrops)	+	Jones, Galbraith, and Al-Hussaini (1962)
Herpes keratitis	? hourly (human amnion) (eyedrops)	±	Tommila (1963)
Herpes kreatitis	c 10 <sup>5</sup> units × 5/day (human leukocyte) (Eyedrops)	+	Kobza et al. (1975)
Herpes keratitis	10 <sup>7</sup> units/ml daily × 7 days (human leukocyte)	+	Jones et al. (1976)
Herpes keratitis	2 to 6 × 10 <sup>4</sup> units/ml × 2/day (human leukocyte) (Eyedrops)	-	Kaufman et al. (1976)
Herpes keratitis	6 × 10 <sup>4</sup> units/ml 3 times/day (human leukocyte) (eyedrops)	±	Sundmacher et al. (1976c)
Herpes keratitis	c 6 × 10 <sup>6</sup> units/ml 3 times/day (human leukocyte) (eyedrops)	+	Pallin, Lundmark, and Brege (1976)

Table 18 (continued)

Antiviral

Disease	
Herpes keratitis	3 × 10 <sup>4</sup> 2 drops (human (eyedra
Genital herpesvirus lesions	4000 u (human (ointm
Genital warts (condylomata acuminata)	4000 u (human (ointm
<b>2. Generalized infections</b>	
Herpesvirus zoster	1 to 3 week (im
Herpesvirus zoster	3 × 1 (hum
Herpesvirus zoster	10 <sup>6</sup> u (hum
Disseminated herpes simplex	c 5 × + 10 <sup>6</sup> u (hum
Disseminated herpes simplex	c 12 × days
Cytomegalovirus inclusion disease	c 5 × (hum am
Cytomegalovirus inclusion disease	c 10 <sup>5</sup> days
Cytomegalovirus inclusion disease	c 2 × (hum
Cytomegalovirus inclusion disease	c 30 (hum
Cytomegalovirus complicating bone marrow transplantation	c 10 (hum
Chronic hepatitis-B	6 u
Chronic hepatitis-B	c 1

Table 18 (continued)

Antiviral Activities of Interferons in Man

Scientific Committee on Interferon (1962)

Scott *et al.* (1977 b, c)

Scientific Committee on Interferon (1965)

Tyrrell and Reed (1973)

Merigan *et al.* (1973 a)

Soloviev (1967, 1969)

Merigan *et al.* (1973 a)

Connolly, Dick, and Field (1962)

Soos *et al.* (1972)Jones, Galbraith, and Al-Hussaini (1962)  
Tommila (1963)Kobza *et al.* (1975)Jones *et al.* (1976)Kaufman *et al.* (1976)Sundmacher *et al.* (1976c)

Pallin, Lundmark, and Brege (1976)

Disease	Treatment <sup>a</sup>	Effect <sup>b</sup> demonstrated	References
Herpes keratitis	$3 \times 10^6$ units/ml 2 drops, 2 times/day (human leukocyte) (eyedrops)	+	Sundmacher, Neumann-Haefelin, and Cantell (1976 a, b)
Genital herpesvirus lesions	4000 units/GM (human leukocyte) (ointment)	+	Ikic <i>et al.</i> (1974; 1975 c)
Genital warts (condylomata acuminata)	4000 units/GM (human leukocyte) (ointment)	+	Ikic <i>et al.</i> (1975 a, b)
2. Generalized infections			
Herpesvirus zoster	1 to $3 \times 10^6$ units 3 times/week (human leukocyte) (im)	+	Strander <i>et al.</i> (1973)
Herpesvirus zoster	$3 \times 10^7$ units/day 2-5 days (human leukocyte) (im)	+	Jordan, Fried, and Merigan (1974); Merigan <i>et al.</i> (1977)
Herpesvirus zoster	$10^6$ units/day 5-8 days (human leukocyte) (im)	+	Emodi <i>et al.</i> (1975 b)
Disseminated herpes simplex	c $5 \times 10^5$ units/day $\times$ 7 days + $10^6$ units/day $\times$ 14 days (human leukocyte) (im)	+	Kobza <i>et al.</i> (1975)
Disseminated herpes simplex	c $12 \times 10^5$ units/day $\times$ 2 days + $6 \times 10^5$ units/day $\times$ 4 days (human leukocyte) (intrathecal)	-	Declercq <i>et al.</i> (1975)
Cytomegalovirus inclusion disease	c $5 \times 10^4$ unit/day $\times$ 2 weeks (human leukocyte and amnion) (iv)	$\pm$	Falcoff <i>et al.</i> (1966)
Cytomegalovirus inclusion disease	c $10^5$ to $10^6$ units/ml 8-10 days (human leukocyte) (im)	+	Emodi <i>et al.</i> (1976)
Cytomegalovirus inclusion disease	c $2-4 \times 10^5$ units/kg/day $\times$ 10-14 days (human leukocyte) (im)	$\pm$	Arvin, Yeager, and Merigan (1976)
Cytomegalovirus inclusion disease	c $30 \times 10^6$ units/kg (human leukocyte) (im)	+	Merigan <i>et al.</i> (1977)
Cytomegalovirus complicating bone marrow transplantation	c $10^6$ units/day $\times$ 5 days (human leukocyte) (im)	$\pm$	O'Reilly <i>et al.</i> (1976)
Chronic hepatitis-B	$6 \times 10^3$ to $17 \times 10^4$ units/kg/daily (human leukocyte) (im)	+	Greenberg <i>et al.</i> (1976); Merigan <i>et al.</i> (1977)
Chronic hepatitis-B	c $15-20 \times 10^4$ units/kg/day	+	Desmyter <i>et al.</i> (1976)

Table 18 (continued)

Disease	Treatment <sup>a</sup>	Effect <sup>b</sup> demonstrated	References
	7 times/2 weeks (human fibroblast)		
Chronic hepatitis-B	c $10^7$ units/day $\times$ 14 days (human fibroblast) (im)	+	Kingham <i>et al.</i> (1977)
Congenital rubella Syndrome	c $3 \times 110^6$ units/day $\times$ 2 weeks (human leukocyte) (im)	+	Larsson <i>et al.</i> (1976)
Marburg virus	c $3 \times 10^6$ units/day $\times$ 2 weeks (human leukocyte) (im)	+	Anonymous (1977)
<b>B. Antitumor studies</b>			
Acute leukemia	$\sim 10^3$ units/ml, repeatedly (human leukocyte) (iv)	$\pm$	Falcoff <i>et al.</i> (1966)
Acute leukemia	$1.25-2.5 \times 10^6$ units/day 1 to 12 month (human leukocyte) (im)	?	Ahstrom <i>et al.</i> (1974)
Hodgkin's disease (stage IVB)	c $5 \times 10^6$ units/day $\times 1\frac{1}{2}$ month $7 \times 10^6$ units/day $\times 5\frac{1}{2}$ months (human leukocyte) (im)	+	Blomgren <i>et al.</i> (1976)
Multiple myeloma	c $6 \times 10^6$ units/day (human leukocyte) (im)	+	Strander (1977a, c)
Juvenile larynx papilloma	c ? (human leukocyte) (im)	+	Strander (1977a, c)
Osteosarcoma	c $3 \times 10^6$ units/day $\times$ 1 month + $3 \times 10^6$ units, 3 times/week $\times 17$ months	+	Strander <i>et al.</i> (1974, 1975, 1976, 1977) Strander (1977a, b, c) Adamson <i>et al.</i> (1977)

a Indicated route of inoculation: id = intradermal; in = intranasal; sc = subcutaneous; iv = intravenous; im = intramuscular

b Effect demonstrated: - = no evidence of effect;  $\pm$  = suggestive effect; + = effectiveness reported.

c Uncontrolled trial with only one or two patients.

Volunteers were injected intradermally with 0.1 ml of a preparation of monkey interferon titrating about 2000 units/ml, and vaccinia virus was scarified into the area the next day. A highly significant protection was reported. In more recent trials human fibroblast interferon was also found to be effective in preventing vaccinia "takes" (Scott *et al.*, 1977c) and crude human fibroblast and human leukocyte interferon preparations seemed of similar efficacy (Scott *et al.*, 1977b).

A large trial for prophylaxis against influenza was reported by Soloview (1967) in which volunteers were treated with low-titered human interferon aerosol intranasally 24 hours prior to virus by the same route. Prophylaxis was

apparent from lower several thousand picogram was reported to may be attributable to produce interferon in ducer was also being preparations are commercial prophylaxis and therapy Fried, 1975; G. Svet-

Interferon prophylaxis is considerably less important than rhinoviruses by intranasal without apparent effect. In a study, using human rhinovirus challenge, Ahstrom *et al.* (1973) reported that interferon, orally both 1 day before and 1 day after, significantly prevent symptoms. A 10-fold lower dose of interferon was slight, only delaying the onset of symptoms.

Locally injected monkey interferon was ineffective (Connolly, 1977). The titer of the interferon was low-titered. In a study, using vaccinia inoculations with monkey interferon, Soos *et al.* (1977) found that monkey leukocyte interferon resulted in quicker healing of the lesions.

Vaccinia keratitis was treated with a mixture of interferon and Al-Hussaini (1977). The patients were given 10 drops each half-hourly. The infiltration of the epithelium was reduced at the same rate as seen after 4 days. However, the infiltration was quenched in both debilitated patients.

Herpetic keratitis was treated with interferon hourly (Tommila, 1977). The patients treated by interferon had a faster healing time than the patients treated by iodoacetamide.

More than 10 years ago, a patient with severe herpetic keratitis was treated with topical 4% interferon (4 times/day)<sup>14</sup>. The patient had a rapid healing time.

<sup>14</sup> The concentration of interferon was 4%.

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DN<sub>A</sub> CODING FOR POLY:PEPTIDE WITH INTERFERON ACTIVITY + USEFUL IN PREPN. OF HUMAN INTERFERON IN LARGE AMOUNTS

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(26) Novel DNA, cloned DNA, recombinant plasmid containing the DNA, microorganism containing the recombinant plasmid and process for their production.

(27) The invention relates to a DNA which codes for a polypeptide with interferon activity, a cloned DNA showing complementarity to human interferon mRNA, a recombinant plasmid containing such DNA, a microorganism containing the recombinant plasmid and a process for producing said DNA, said cloned DNA, said recombinant plasmid and said microorganism.

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15 Our Ref.: P 861 EP (Ra/fw) October 30, 1980  
Case: 265-Europ.

20 Juridical Foundation, Japanese Foundation for Cancer  
Research, Tokyo, Japan

25 "Novel DNA, Cloned DNA, Recombinant Plasmid Containing  
the DNA, Microorganism Containing the Recombinant Plasmid  
and Process for their Production"

30 Priorities: October 30, 1980, Japan, No. 139 289/79  
March 19, 1980, Japan, No. 33 931/80

35 The present invention relates to a DNA which codes for a  
polypeptide with interferon activity and to a recombinant  
plasmid containing the DNA. The present invention also  
pertains to a microorganism containing the recombinant  
plasmid.

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Interferon is a glycoprotein (molecular weight approx. 20,000) with antiviral activity, discovered by Isaacs and Lindenmann in 1957. Subsequent studies have indicated antitumor activity of the substance in addition to antiviral activity and hence a wide clinical application of this substance is expected. For instance, it has been reported that interferon may be effectively used to treat various viral diseases, osteosarcoma and mammary carcinoma.

10 However, because of its high species-specificity, only the interferon derived from human cells can be used for human application. At present, the interferon which is being used for administration has a relative activity of about  $10^6$  (International units) per 1 mg, which corresponds to a purity of about 0.1 - 0.01%.

15 Moreover, the use of the interferon is quite limited because of difficulties in mass production. At present only about 1% of the interferon requirement even for clinical tests ( $10^{13}$  units per annum) can be met. For these reasons there is a great need to develop technology to produce human 20 interferon in high purity, with ease and in large quantities.

25 To this end, a novel technique has been developed for producing interferon with ease and in a large quantity by inserting a human interferon gene into a plasmid DNA (for instance plasmid DNA derived from Escherichia coli) with 30 recombinant DNA (deoxyribonucleic acid) technology.

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1 In accordance with the present invention, a DNA  
which codes for a polypeptide with interferon activity is  
prepared using the human interferon messenger RNA as a  
template and a novel recombinant plasmid containing the DNA  
5 is prepared. In addition, the recombinant plasmid may be  
inserted into a host microorganism.

10 The DNA which codes for a polypeptide with  
interferon activity and the recombinant plasmid containing  
the DNA have been obtained for the first time by the present  
inventors. The DNA and the recombinant plasmid may be used,  
15 inter alia, for amplification of human interferon in bacteria  
such as Escherichia coli. Such bacteria are then useful for  
the production of human interferon in large quantities at  
low cost.

15 The DNA and the recombinant plasmid of the present  
invention are prepared by the following general procedure.

First, cytoplasmic RNA is extracted from (1) human  
fibroblast, MG63 cells or others induced by poly(I): poly(C)  
20 (a double stranded RNA composed of polyinosinic acid  
and polycytidyllic acid) or other inducers, (2) human leucocyte,  
lymphoblastic cells, NAMALWA cells or others induced by  
Sendai virus or other inducers, or (3) lymphocytes induced  
by various mitogens or other inducers. From this RNA, the  
25 human interferon messenger RNA (hereinafter messenger RNA)  
is referred to as mRNA) containing poly A (polyadenylic acid)  
is isolated. A double stranded DNA is synthesized, for  
example, by reverse transcriptase, with the mRNA preparation  
having high interferon mRNA activity as a template.  
30 A recombinant is obtained by inserting the synthesized DNA  
into a vector DNA such as Escherichia coli plasmid DNA by  
the technique of in vitro DNA recombination. The recombinant  
is labelled with a radio isotope for use as a probe.  
Recombinant plasmids having an inserted portion which is

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complementary to the human interferon mRNA are selected. The DNA which codes for a polypeptide with interferon activity is recovered from the recombinant plasmid and the base sequence of the DNA is determined.

5

10 Fig. 1 illustrates restriction endonuclease maps of:

15 (a) a gene which shows complementarity to the human fibroblast interferon mRNA in the recombinant #319 used to make a novel recombinant plasmid #319-13; and

15 (b) a gene which shows complementarity to the human fibroblast interferon mRNA in the novel recombinant plasmid #319-13.

20 The present invention relates to a DNA which codes for a polypeptide with interferon activity, a recombinant plasmid containing the DNA and a microorganism containing the recombinant plasmid.

25 The DNA of the present invention may be a cloned DNA showing complementarity to the human interferon mRNA, a cloned DNA which codes for a polypeptide with interferon activity or a cloned DNA which codes for human interferon polypeptide. Especially a DNA which encompasses the entire coding region of the human fibroblast interferon mRNA is a preferred example of the DNA of the present invention.

30 The recombinant plasmid of the present invention is a recombinant plasmid wherein the DNA mentioned above is inserted in a vector DNA such as pBR322, pCR1, pMB9 or pSC1.

35 The recombinant plasmids named #319 and #319-13 are preferred examples of a recombinant plasmid according to the invention.

The DNA and the recombinant plasmid are inserted in a host microorganism and the transformant can be used to

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produce a substance having interferon activity.

As the host microorganism, Escherichia coli X1776 is preferably used.

5 An example of the processes of producing the DNA, the recombinant plasmid and the transformant of the present invention is as follows.

10 First, human fibroblasts may be obtained from fetus-derived foreskin, or the like. A small amount of interferon is then added to a culture fluid of human fibroblasts to prime the interferon synthesis by human fibroblasts, to which poly(I): poly(C) is added to induce the synthesis of interferon mRNA. Cycloheximide is added simultaneously to increase the level of interferon mRNA. At an appropriate time (about 4 hours) after the human fibroblasts have been superinduced in the above manner, cells are collected and destroyed and the nuclei are removed. Cytoplasmic total RNA is extracted with phenol, or the like. The RNA can also be extracted by destroying the whole cells, extracting both DNA and RNA with, for example, phenol, and degrading and removing the DNA with DNase.

15

20

25 Further, interferon mRNA can also be extracted from MG63 cells induced by poly(I): poly(C) or other inducers, human leucocyte or lymphoblastic cells induced by Sendai virus or other inducers, and lymphocytes induced by various mitogens or other inducers.

30 The thus extracted RNA is dissolved in a salt solution of NaCl or KCl at a high concentration such as 0.5M and put on a column of oligo (dT) cellulose to adsorb mRNA having poly(A) on the column. Elution is carried out with water, a salt solution at a low concentration such as 10 mM Tris-HCl buffer, or the like to isolate mRNA having poly(A).

35 The isolated mRNA is fractionated by sucrose density gradient centrifugation. Interferon mRNA activity in each fraction is checked by determining interferon activity (antiviral activity) of the protein which is synthesized in oocytes of African claw toad (Xenopus laevis) after micro-injecting a part of the mRNA in each fraction. The determination of interferon activity is carried out according to the

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method described in Japan J. Microbiol. 18, 449-456, (1974).

Then, a DNA showing complementarity to the mRNA is synthesized in vitro by a reverse transcriptase, which is obtained from avian myeloblastosis virus, using, as the template, an mRNA having the highest interferon mRNA activity.

5 The synthesis is carried out as follows.

An mRNA is reacted at an appropriate temperature (e.g. 37°C) for an appropriate period (e.g. 60 min.) with 10 oligo(dT), MgCl<sub>2</sub> (e.g. 5 mM), NaCl (e.g. 30 mM), mercaptoethanol (e.g. 5 mM) and Tris-HCl buffer (e.g. pH 8.0, 40 mM) using a reverse transcriptase together with deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP) (e.g. 0.5 mM each) as substrates.

15 The thus obtained reaction product is subjected to deproteinization with, for example, phenol, and the template RNA is removed by alkali or ribonuclease treatment. A double stranded DNA is synthesized by a reverse transcriptase in a similar way as the synthesis of the DNA showing 20 complementarity to mRNA described above except that mRNA is replaced by DNA and oligo(dT) is omitted.

25 By using Escherichia coli DNA polymerase I which can be obtained from Escherichia coli MRE 600, or the like, instead of reverse transcriptase, the same double stranded DNA can be synthesized.

After the double stranded DNA which is synthesized by the above described procedure is treated with Nuclease S<sub>1</sub> which can be obtained from Aspergillus oryzae in the presence of ZnCl<sub>2</sub> (e.g. 1 mM), sodium acetate buffer (e.g. 30 0.1 M, pH 4.5), NaCl (e.g. 0.2 M), etc., deoxyadenine chains are formed at both 3' ends of the synthesized DNA by incubating with a terminal transferase purified from calf thymus in the presence of potassium cacodylate buffer (e.g. pH 7.6, 0.14 M), Tris (base) (e.g. 0.03 M), dithiothreitol (e.g. 35 0.1 mM), CoCl<sub>2</sub> (e.g. 1 mM) and dATP (e.g. 1 mM) at an appropriate temperature (e.g. 37°C) for an appropriate period (e.g. 20 min.)

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On the other hand, a plasmid DNA which is used as a vector DNA, e.g. Escherichia coli plasmid pBR322 DNA [Gene vol. 2, p. 95-113 (1977)], is cleaved at one site by treating with a restriction endonuclease EcoRI, which can be obtained, for example, from Escherichia coli RY13, in the presence of Tris-HCl buffer (e.g. pH 7.5, 10 mM),  $MgCl_2$  (e.g. 6 mM), NaCl (e.g. 0.1 M), mercaptoethanol (e.g. 6 mM), or the like and then treated with phage  $\lambda$ -derived exonuclease, which can be obtained, for example, from Escherichia coli W3102 ( $\lambda$  cI851) x 13, in the presence of Na-glycine buffer (e.g. pH 9.5, 0.1 M),  $MgCl_2$  (e.g. 5 mM), or the like. Thereafter deoxythymidine chains are formed at both 3' ends in the same way as for the above-described synthesized double stranded DNA by using dTTP instead of dATP.

Synthetic double stranded DNA and plasmid DNA which are chain-elongated at both 3' ends as described above are incubated at an appropriate temperature for an appropriate period with Tris-HCl buffer (e.g. pH 7.5, 50 mM), NaCl (e.g. 0.1 M), EDTA (e.g. 5 mM), or the like and hybridized with hydrogen bonds formed by adenine and thymine. Then, a transformable Escherichia coli strain, e.g. Escherichia coli x1776 (Molecular Cloning of Recombinant DNA, Scott, W. A. & Werner, R., edited, Academic Press p. 99-114, 1977) is transformed with the hybridized DNA by the method of Enea et al. (J. Mol. Biol. vol. 96, p. 495-509, 1975) or the like.

In the novel recombinant plasmid DNA thus obtained, there exists a vector DNA gene, e.g.  $\beta$ -lactamase (enzyme that destroys ampicillin) gene, of Escherichia coli plasmid pBR322. Therefore, the transformed Escherichia coli shows resistance to ampicillin. The following technique is used to pick up a strain with a novel recombinant having a gene which shows complementarity to the human interferon messenger RNA among these ampicillin resistant strains.

First,  $[^{32}P]$  labelled DNA is synthesized with the RNA having interferon mRNA activity described above as a template and the DNA is hybridized with mRNA extracted, without induction by poly(I): poly(C) (therefore, interferon mRNA synthesis is not induced), from the human fibroblasts

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by incubating at a high temperature (e.g. 65°C) in a reaction mixture containing, for example NaCl (e.g. 0.5 M). Then, the hybridized DNA (Probe A) and non-hybridized DNA (Probe B) are separated by hydroxyapatite column chromatography.

5 Next, filter-fixed DNAs of transformants are hybridized separately with Probe B or Probe A according to the technique of Grunstein-Hogness (Proc. Nat. Acad. Sci. USA, vol. 72, p. 3961-3965, 1975) and strains having a DNA hybridizable with Probe B but not or barely with Probe A are discerned

10 by autoradiography.

Then, plasmid DNA is isolated from each of the discriminated strains and hybridized with mRNA having interferon mRNA activity by incubating at a high temperature (e.g. 53°C) in the presence of 80% (w/v) formamide, 0.4 M NaCl, etc. Since the mRNA hybridized with cDNA portion of the plasmid DNA from the above-described strains can be retained on a nitrocellulose filter, whereas unhybridized mRNA can not under certain conditions (refer to example below and Nygaard, A.P. & Hall, B.C., Biophys. Res. Commun. Vol. 12, p. 95-104, 1963); this mRNA can be recovered selectively from the filter at a high temperature (e.g. 60°C) in a solution such as 90% (v/v) formamide and thereafter injected into oocytes of Xenopus laevis.

When interferon is synthesized in the oocytes, 25 the DNA used for hybridization must contain a DNA which is complementary to interferon mRNA; and by this method, a recombinant plasmid DNA having a gene showing complementarity to the human fibroblast interferon mRNA can be isolated.

The recombinant plasmid DNA obtained above or 30 segments cleaved with a restriction endonuclease are labelled with a radio isotope such as <sup>32</sup>P by the Nick translation method (Rigby, et al., J. Mol. Biol. vol. 113, p. 237-251, 1977), or the like, and used as a probe to obtain Escherichia coli strains containing a recombinant plasmid having the interferon mRNA sequence from the above ampicillin resistant strains in the same way as described above. Several strains thus obtained are cultured and the plasmid DNA is isolated therefrom. The plasmid DNA is cleaved with a restriction

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plasmids are also expected to be useful for the mass production of interferon.

One specific embodiment of the present invention is illustrated by the following representative example.

5

Example

After priming of [REDACTED] by overnight incubation with MEM culture medium (product of Nissui Seiyaku Co., Ltd., Japan) containing human interferon which is prepared according to the method described in Proc. Nat. Acad. Sci. USA, 73, 520-523 (1976) (25 U/ml), the fibroblasts were superinduced by adding 10  $\mu$ g/ml of poly(I): poly(C) (product of Galbiochem Co., USA) and 5  $\mu$ g/ml of cycloheximide to the medium. The priming and superinduction are carried out according to the methods described in Brit. J. Exp. Path., 39, 452-458 (1958) and Antimicrob. Agents Chemother., 2, 476-484 (1972), respectively.

After 4 hours,  $1.5 \times 10^9$  superinduced human fibroblasts were destroyed by Teflon homogenizer (sold by Takashima Shoten Co., Japan) at a temperature of 0 to 4°C in the presence of 0.3% NP-40 (product of Daiichi Kagaku Co., Japan) and 50  $\mu$ g/ml heparin in RSB buffer (10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 1.5 mM  $MgCl_2$ ). Nuclei were removed by centrifugation at 3000 rpm and 4°C for 10 minutes and 9.6 mg of cytoplasmic RNA was obtained by extraction 3 times with phenol.

The cytoplasmic RNA was precipitated with 67% ethanol in the presence of 0.1M NaCl, dissolved in 10 ml of 1 mM EDTA solution and incubated at 65°C for 2 minutes. Then, 2.5 ml of a salt solution at a high concentration (0.5 M Tris-HCl, pH 7.5; 1 M NaCl; 50 mM EDTA) was added to the above solution and the mixture was put on a column packed with 0.15 g of an oligo(dT) cellulose (product of P-L Biochemicals Co., USA) to adsorb mRNA containing poly(A). Elution was then carried out with a salt solution at a low concentration (10 mM Tris-HCl, pH 7.5) and water to isolate 250  $\mu$ g of mRNA containing poly(A).

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The mRNA was precipitated with 67% ethanol in the presence of 0.1M NaCl and dissolved in 0.5 ml of 1 mM EDTA solution. The solution was incubated at 65°C for 2 minutes, subjected to centrifugation through a 5 - 25% sucrose-density gradient containing 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl and 1 mM EDTA (rotated at 35,000 rpm using the SW40 rotor of Beckmann Co., U.S.A.) at 4°C for 16 hrs. and fractionated into 20 fractions.

The interferon mRNA activity of each of these fractions was determined as mentioned above, and the results are shown in Table 1 below.

Table 1

Fraction No.	Interferon Activity
9	< 50 units/ml
10	44
11	550
12	52

The mRNA in Fraction No. 11 was approximately 5 µg. This mRNA and a reverse transcriptase were incubated at 37°C for an hour in 20 µl of a reaction mixture consisting of 5 µg mRNA; 0.5 mM dATP; 0.5 mM dTTP; 0.5 mM dGTP; 0.5 mM dCTP; 1 µg oligo(dT) (product of P-L Biochemicals Co., USA); 8 units reverse transcriptase (derived from Avian myeloblastosis virus, for example, product of Life Science Inc. Florida, USA); 5 mM MgCl<sub>2</sub>; 30 mM NaCl; 5 mM mercaptoethanol; and 40 mM Tris-HCl (pH 8.0) and then deproteinized with phenol. After RNA was removed by treatment with 0.3 N NaOH at 37°C for 15 hours, the synthesized single stranded DNA was incubated at 37°C in 20 µl of a reaction mixture [the same mixture as described above except that mRNA and oligo(dT) were omitted] for one hour to synthesize 1.5 µg of a double stranded DNA.

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The double stranded DNA was treated with Nuclease S<sub>1</sub> (product of Bethesda Research Laboratories Inc., USA which is referred to as BRL, hereinafter) in 50  $\mu$ l of a reaction mixture (1.5  $\mu$ g double stranded DNA; 1 mM ZnCl<sub>2</sub>; 5 0.1 M sodium acetate, pH 4.5; 0.2 M NaCl; 0.05 unit S<sub>1</sub>) at 37°C for 30 minutes and the enzyme was removed by phenol extraction. The DNA was precipitated with ethanol and then treated with a terminal transferase in 20  $\mu$ l of a reaction mixture consisting of 1.5  $\mu$ g DNA; 0.14 M potassium cacodylate, 10 pH 7.6; 0.03 M Tris (base); 0.1 mM dithiothreitol; 1 mM CoCl<sub>2</sub>; 1 mM dATP; and 1 unit terminal transferase (product of BRL) at 37°C for 20 minutes to obtain about 1.5  $\mu$ g of a product wherein 100 deoxyadenosine chains were formed at both 3' ends of the double-stranded DNA.

15 In an alternative procedure, 10  $\mu$ g of Escherichia coli plasmid pBR322 DNA (product of BRL) was treated at 37°C for 2 hours with a restriction endonuclease EcoRI in 100  $\mu$ l of a reaction mixture consisting of 10 mM Tris-HCl, pH 7.5; 6 mM MgCl<sub>2</sub>; 0.1 M NaCl; 6 mM mercaptoethanol; and 10 units 20 EcoRI (product of BRL) leading to cleavage at the only cutting site in pBR322 DNA. The cut plasmid DNA was treated with an exonuclease derived from phage  $\lambda$  in 200  $\mu$ l of a reaction mixture consisting of 10  $\mu$ g DNA; 0.1 M Na-glycine, pH 9.5; 5 mM MgCl<sub>2</sub>; 50  $\mu$ g/ml albumin (product of 25 Merck & Co., USA); and 17.5 units  $\lambda$  exonuclease (product of Miles Laboratories Inc., USA) at 0°C for 90 minutes and the enzyme was removed by phenol extraction. The DNA was treated with a terminal transferase in 50  $\mu$ l of a reaction mixture [10  $\mu$ g DNA; 0.14 M potassium cacodylate, pH 7.6; 0.03 M Tris 30 (base); 0.1 mM dithiothreitol; 1 mM CoCl<sub>2</sub>; 1 mM dTTP; 2 units terminal transferase] at 37°C for 20 minutes to obtain about 0.5  $\mu$ g of a product wherein 100 deoxythymidine chains were formed at both 3' ends of plasmid pBR322 DNA described above.

35 Then, 0.02  $\mu$ g of the synthesized double stranded DNA obtained above, and 0.1  $\mu$ g of the plasmid pBR322 DNA were incubated for hybridization in a solution containing 0.1 M NaCl, 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA at 65°C

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The double stranded DNA was treated with Nuclease S<sub>1</sub> (product of Bethesda Research Laboratories Inc., USA which is referred to as BRL, hereinafter) in 50  $\mu$ l of a reaction mixture (1.5  $\mu$ g double stranded DNA: 1 mM ZnCl<sub>2</sub>; 0.1 M sodium acetate, pH 4.5; 0.2 M NaCl; 0.05 unit S<sub>1</sub>) at 37°C for 30 minutes and the enzyme was removed by phenol extraction. The DNA was precipitated with ethanol and then treated with a terminal transferase in 20  $\mu$ l of a reaction mixture consisting of 1.5  $\mu$ g DNA; 0.14 M potassium cacodylate, pH 7.6; 0.03 M Tris (base); 0.1 mM dithiothreitol; 1 mM CoCl<sub>2</sub>; 1 mM dATP; and 1 unit terminal transferase (product of BRL) at 37°C for 20 minutes to obtain about 1.5  $\mu$ g of a product wherein 100 deoxyadenosine chains were formed at both 3' ends of the double-stranded DNA.

In an alternative procedure, 10  $\mu$ g of Escherichia coli plasmid pBR322 DNA (product of BRL) was treated at 37°C for 2 hours with a restriction endonuclease EcoRI in 100  $\mu$ l of a reaction mixture consisting of 10 mM Tris-HCl, pH 7.5; 6 mM MgCl<sub>2</sub>; 0.1 M NaCl; 6 mM mercaptoethanol; and 10 units EcoRI (product of BRL) leading to cleavage at the only cutting site in pBR322 DNA. The cut plasmid DNA was treated with an exonuclease derived from phage  $\lambda$  in 200  $\mu$ l of a reaction mixture consisting of 10  $\mu$ g DNA; 0.1 M Na-glycine, pH 9.5; 5 mM MgCl<sub>2</sub>; 50  $\mu$ g/ml albumin (product of Merck & Co., USA); and 17.5 units  $\lambda$  exonuclease (product of Miles Laboratories Inc., USA) at 0°C for 90 minutes and the enzyme was removed by phenol extraction. The DNA was treated with a terminal transferase in 50  $\mu$ l of a reaction mixture (10  $\mu$ g DNA; 0.14 M potassium cacodylate, pH 7.6; 0.03 M Tris (base); 0.1 mM dithiothreitol; 1 mM CoCl<sub>2</sub>; 1 mM dTTP; 2 units terminal transferase) at 37°C for 20 minutes to obtain about 0.5  $\mu$ g of a product wherein 100 deoxythymidine chains were formed at both 3' ends of plasmid pBR322 DNA described above.

Then, 0.02  $\mu$ g of the synthesized double stranded DNA obtained above, and 0.1  $\mu$ g of the plasmid pBR322 DNA were incubated for hybridization in a solution containing 0.1 M NaCl, 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA at 65°C

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for 2 minutes, at 45°C for one hour, at 37°C for one hour and at room temperature for one hour. Then, Escherichia coli X1776 was transformed with the hybridized recombinant following the method of Enca et al.

5        About 4,000 ampicillin-resistant strains were isolated by this method. 3,600 resistant strains were chosen, and the DNA of each strain was fixed on nitrocellulose filters in duplicate (Grunstein-Hogness Method).

10      In a further procedure, [<sup>32</sup>P] labelled single stranded DNA was synthesized (about 0.44  $\mu$ g, specific radioactivity approx.  $6 \times 10^8$  c.p.m./ $\mu$ g) by reverse transcriptase in the same way as that for single stranded DNA mentioned above (dCTP was labelled with <sup>32</sup>P) using the interferon mRNA fraction (about 10  $\mu$ g) which had been extracted and partially 15 purified as described above, as a template. The DNA was hybridized in 50  $\mu$ l of a reaction mixture (25  $\mu$ g mRNA; 0.45  $\mu$ g single stranded DNA labelled with <sup>32</sup>P; 0.5 M NaCl; 25 mM Pipes buffer, pH 6.5) at 65°C for 40 hours with 25  $\mu$ g of mRNA extracted from human fibroblasts which had not been 20 induced by poly(I): poly(C). The latter mRNA was prepared by the same method used to extract poly(I): poly(C)-induced mRNA. The reaction mixture was put on a column packed with 0.2 g of a hydroxyapatite, and elution was first carried out with 5 ml of 0.14 M phosphate buffer (pH 6.5) to elute the 25 single stranded DNA, and then with 5 ml of 0.4 M phosphate buffer to elute the DNA hybridized with RNA. As a result, the DNA (about 90% of the total) (Probe A) which hybridized with the mRNA mentioned above, and the DNA (about 10% of the total) (Probe B) which did not hybridize were isolated.

30      Each probe was then hybridized separately with the above DNA fixed on the nitrocellulose filters according to the Grunstein-Hogness method. Four strains were identified which reacted mainly to Probe B but little to Probe A under autoradiography.

35      Table 2 shows the extent of reaction of the DNAs from the four strains to each probe as revealed by autoradiogram.

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Table 2

Ampicillin-resistant strains	Extent of Reaction of Probe with DNA in the strains	
	Probe A	Probe B
# 319	++	++++
# 644	+	+++
# 746	-	++
#3578	+	+++-+

Plasmid DNA was isolated from cells of the four strains by the method of Currier and Nester (Analyst. Biochem. vol. 76, p. 431-441, 1976). Then, these DNAs were hybridized with the interferon mRNA as follows.

First, 5  $\mu$ g of plasmid DNA was linearized by incubating with restriction endonuclease Hind III, which can be obtained from Haemophilus influenzae Rd, in 50  $\mu$ l of a reaction mixture consisting of 10 mM Tris-HCl, pH 7.5; 6 mM  $MgCl_2$ ; 50 mM NaCl; 6 mM mercaptoethanol; and 5 units Hind III (product of BRL) at 37°C for 2 hours. After deproteination by phenol extraction, the DNA was precipitated with ethanol and dissolved in 20  $\mu$ l of 80% (w/v) formamide. The solution was denatured at 85°C for 10 minutes and was then incubated in a solution consisting of 2.5  $\mu$ g mRNA, 20  $\mu$ l 80% (w/v) formamide, 20 mM Pipes buffer (pH 6.5), 0.4 M NaCl and 5 mM EDTA, at 53°C. Four hours later the mixture was mixed with 0.4 ml of 3 x SSC (1 x SSC corresponds to 0.15 M NaCl, 0.015 M sodium citrate) at 0°C, and was filtered through a nitrocellulose filter (diameter : 1 cm, pore size : 0.45  $\mu$ m) at a rate of about 0.5 ml per minute. After washing the filter with about 1.5 ml of 2 x SSC, the filter was immersed in a solution consisting of 0.6 ml of 90% (v/v) formamide, 20 mM Pipes buffer, 0.1% SDS (sodium dodecylsulfate) and 5 mM EDTA. Incubation of the filter at 60°C for 2 minutes and the removal of the solution were repeated 3 times and the RNA eluted from the nitrocellulose filter into the solution (1.8 ml) was precipitated with ethanol in the presence of

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0.1 M NaCl. The mRNA containing poly(A) was isolated from the RNA by using oligo(dT) cellulose column chromatography, dissolved in a mixture of 3  $\mu$ l of 10 mM Tris-HCl (pH 7.5) and 88 mM NaCl and injected into the oocytes of Xenopus laevis. After 15 hours the interferon synthesized in the oocytes was determined (antiviral activity).

Table 3 shows the interferon mRNA activity of the mRNA which has hybridized with the DNA derived from the four bacterial strains mentioned above.

10

Table 3

	Bacterial strain	Interferon mRNA activity (unit/ml)
15	# 319	360
	# 644	< 10
	# 746	15
	#3578	< 10
20	pBR322DNA	< 10

20

Five  $\mu$ g of plasmid DNA obtained from strain #319 by the Currier and Nester method was cleaved with restriction endonuclease Hind III in the same manner as mentioned above. The DNA and the recombinant plasmid 8GpBR322 DNA (the vector was pBR322) (obtained from the Institute for Molecular Biology I of University of Zurich or prepared by the method described in Nature 281, 40-46, 1979) containing rabbit  $\beta$ -globin gene, separately or as a mixture, were hybridized with a mixture of rabbit globin mRNA (obtained from rabbit red blood cells) (1  $\mu$ g) and interferon mRNA (2.5  $\mu$ g) obtained from human fibroblasts under the same conditions as mentioned above. The mRNA which formed a hybrid was injected into the oocytes of Xenopus laevis. The oocytes were then incubated for 15 hours in Barth's culture medium (J. Embryol. Exp. Morph. 7, 210, 1959) containing [ $^3$ H] labelled histidine, and [ $^3$ H] labelled globin was isolated by acrylamide gel electrophoresis and determined quantitatively by fluorography

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according to the method described in Eur. J. Biochem. 46, 83-88, (1974). The interferon was determined by antiviral activity as described above. The synthesis of rabbit  $\beta$ -globin and the human interferon was determined in this way.

5 The result is shown in Table 4 below.

Table 4

	D N A	Synthesized interferon activity	Amount of globin synthesized
10	# 319	200 (units/ml)	-
	BGpBR322	35	+++
15	mixture of both plasmids	160	++

From the results of this experiment it has been established that DNA of #319 has DNA (the interferon gene) which forms a hybrid specifically with the interferon mRNA.

20 The DNA of #319 was cleaved with several restriction endonucleases and a restriction endonuclease map, Fig. 1(a), was made by agarose electrophoresis.

25 Restriction endonucleases, Pst I, Bgl II and Hind III (sold by BRL, etc.) cleave #319 DNA at the sites illustrated in Fig. 1 (a).

30 The segments obtained by cleaving #319 DNA with restriction endonucleases Pst I and Bgl II were isolated and purified by gel electrophoresis according to the method of Tabak & Flavell (Nucleic Acids Research, vol. 5, p. 2321-2332, 1978). The segments were labelled with  $^{32}P$  according to the method of Rigby, et al. (J. Mol. Biol. vol. 113, p. 237-251, 1977) and the labelled segment was used as a probe. Several strains containing a plasmid which shows complementarity to the probe were isolated from the above 35 ampicillin-resistant strains according to the above method of Grunstein & Hogness (Proc. Nat. Acad. Sci. U.S.A., vol. 72, p. 3961-3965, 1975), namely the colony hybridization method. Plasmid DNAs were obtained from each of the strains according

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to the above method of Currier-Nester and the inserted portions thereof were cleaved with a restriction endonuclease such as Hind III. The cut plasmid DNA segments were compared in length and the longest plasmid DNA segment was selected.

5 The plasmid was named #319-13.

10 The restriction endonuclease map of the plasmid is illustrated in Fig. 1 (b), which substantiates that the novel plasmid has an mRNA sequence containing the mRNA sequence of #319. Primary structure (base sequence) of the mRNA sequence inserted in the plasmid of #319-13 was determined by the method of Maxam-Gilbert (Proc. Nat. Acad. Sci, U.S.A. vol. 74, p. 560-564, 1977). The primary structure is given in Table 5 below.

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Table 5

The DNA sequence permits prediction of the entire amino acid sequence for human fibroblast interferon (amino acids 1-166) and its putative signal peptide (amino acids -21 to -1) as shown in the line above the DNA sequences.

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It is important that in the sequence there exist without any errors the base sequence (three base pairs) corresponding to the amino acid sequence from the amino-terminal to 13th amino acid of the human fibroblast interferon reported by Knight, et al. (Science vol. 207, p. 525-526, 1980). This fact establishes that the #319-13 plasmid of the present invention has the human fibroblast interferon mRNA sequence.

Further, it is apparent from the data of the primary sequence that the plasmid encompasses the entire coding region of the protein of the above mRNA and probably the coding region of the signal peptide.

Therefore, transformation of the plasmid or mRNA inserted therein to other expression plasmids enables a host such as Escherichia coli to produce interferon. For such purposes, the #319-13 plasmid which is named TpIF 319-13, transformed in Escherichia coli X1776, has been deposited with the American Type Culture Collection, Rockville, Maryland, U.S.A. under accession number ATCC 31712 and is freely available to the public.

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What is Claimed is

1. A DNA which codes for a polypeptide with interferon activity.
2. A cloned DNA showing complementarity to human interferon messenger RNA.
3. The cloned DNA according to claim 2, wherein the messenger RNA is human fibroblast interferon messenger RNA.
4. A cloned DNA which codes for human interferon polypeptide.
5. The cloned DNA according to claim 4, wherein the polypeptide is human fibroblast interferon polypeptide.
6. A recombinant plasmid wherein a DNA showing complementarity to human interferon messenger RNA is inserted in a vector DNA.
7. The recombinant plasmid according to claim 6, wherein the messenger RNA is human fibroblast interferon messenger RNA.
8. The recombinant plasmid according to claims 6 or 7, wherein the plasmid is an Escherichia coli plasmid.
9. The recombinant plasmid according to claim 8, wherein the plasmid is selected from pBR322, pCRL, pMB9 and pSCL.
10. The recombinant plasmid TpIF 319-13.
11. A microorganism containing the recombinant plasmid defined in claim 6.
12. A microorganism containing the recombinant plasmid defined in claim 7.
13. The microorganism according to claim 11 which is Escherichia coli X1776.

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14. The microorganism according to claim 12 which is Escherichia coli X1776.

15. Escherichia coli X1776/TpIF 319-13 ATCC 31712.

16. A process for producing a DNA which codes for a polypeptide with interferon activity by recombinant DNA technology.

17. The process according to claim 16, wherein the polypeptide is the human fibroblast interferon polypeptide.

18. A process for producing a DNA which codes for a polypeptide with interferon activity by using human interferon messenger RNA as a template.

19. The process according to claim 18, wherein the DNA is a cloned DNA showing complementarity to human interferon messenger RNA.

20. The process according to claim 19, wherein the messenger RNA is human fibroblast interferon messenger RNA.

21. A process for producing a recombinant plasmid, which comprises inserting a DNA showing complementarity to human interferon messenger RNA in a vector DNA.

22. The process according to claim 21, wherein the messenger RNA is human fibroblast interferon messenger RNA.

23. The process according to claim 21 or 22, wherein the vector DNA is an Escherichia coli plasmid.

24. The process according to claim 23, wherein the plasmid is selected from pBR322, pCR1, pMB9 and pSC1.

25. The process according to claim 21, wherein the recombinant plasmid is TpIF 319-13.

26. A process for producing a microorganism containing a recombinant plasmid defined in claim 21 or 22, which comprises transforming a microorganism with said

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recombinant plasmid in a conventional manner.

27. The process according to claim 26, wherein the microorganism is Escherichia coli X1776.

28. The process according to claim 26, wherein the recombinant plasmid is TpIF 319-13.

29. The process according to claim 26, wherein the microorganism containing a recombinant plasmid is Escherichia coli X1776 TpIF 319-13 ATCC 31712.

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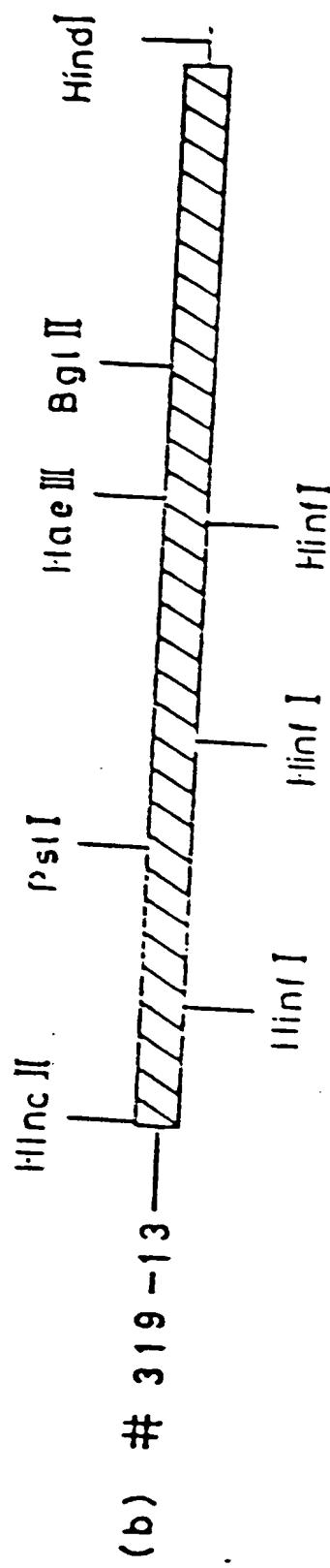
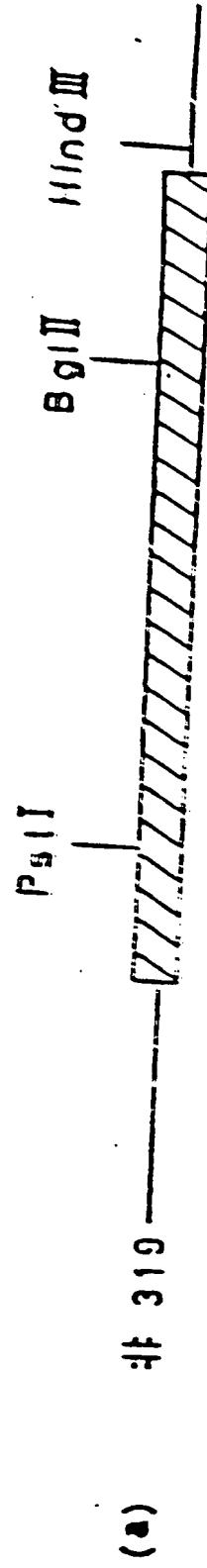
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Fig. 1



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Enclosure 8

SHEET 1 OF 2

INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)				ATTY. DOCKET NO. 2083.2	SERIAL NO. 06/564,224		
				APPLICANT David F. Mark et al.			
				FILING DATE December 20, 1983	GROUP 174		
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EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE	
BB	4 4 1 4 1 5 0	11/8/83	Goeddel	424	85		
AB							
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AG							
AH							
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AJ							
AK							
FOREIGN PATENT DOCUMENTS							
	DOCUMENT NUMBER	PUBLICATION DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES NO	
BB	AL 0 0 4 2 2 4 6	12/23/81	Europe	—	—		
BB	AM 0 0 2 8 0 3 3	5/6/81	Europe (Juridical Foundation Foundation for Cancer Research)	Japanese			
BB	AN 0 0 4 1 3 1 3	12/9/81	Europe (Biogen N.V.)				
BB	AU						
BB	AP						
OTHER DISCLOSURES (Including Author, Title, Date, Pertinent Pages, Place of Publication, Etc.)							
BB	AR	Glossary of Cytogenetics and Cytogenetics, 4th Ed., p. 381, Springer-Verlag (1976)					
BB	AS	Bleopard, H.M., et al., Nature (1981), 294:563-565					
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EXAMINER					DATE CONSIDERED		
Blair Dozal					12-17-85 TPL 04-1		

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SHEET 2 OR 2

INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)			ATTY. DOCKET NO. 2083.2	SERIAL NO. 06/564,224
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			FILING DATE December 20, 1983	GROUP 174
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EXAMINER <i>Blanche Hazel</i>			DATE CONSIDERED <i>12-17-85</i>	
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(58) Field of search

C3H

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(54) Production of interferon by  
 genetic engineering

(57) A process to isolate genetic material (DNA) containing the nucleotide sequence coding for interferon in human fibroblastic cells which comprises cultivating cells producing interferon when exposed to an inducer of interferon, exposing same to such inducer, extracting messenger RNA from said induced cells, purifying the interferon messenger RNA, transcribing the messenger RNA into DNA and cloning the DNA in a suitable vector is described. Preferred cells are human diploid foreskin cells. In addition a process for engineering a bacterial strain to produce interferon polypeptide which comprises introducing a cloned interferon DNA into a suitable vector-carrier is specified. A preferred vector-carrier is *E. coli*. There is also provided the mRNA of human interferon in highly purified form, to the mRNA of human interferon in  $\beta 1$  highly purified form, to the mRNA of human interferon in  $\beta 2$  highly purified form, to the DNA coding for a polypeptide having interferon activity, insertable in a vector, such as plasmid pBR322, and also to human interferon  $\beta 1$  in highly purified form, and human interferon  $\beta 2$  in highly purified form.

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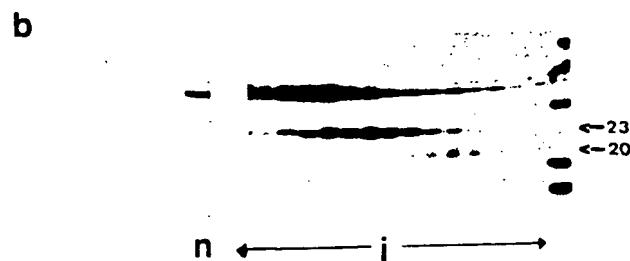
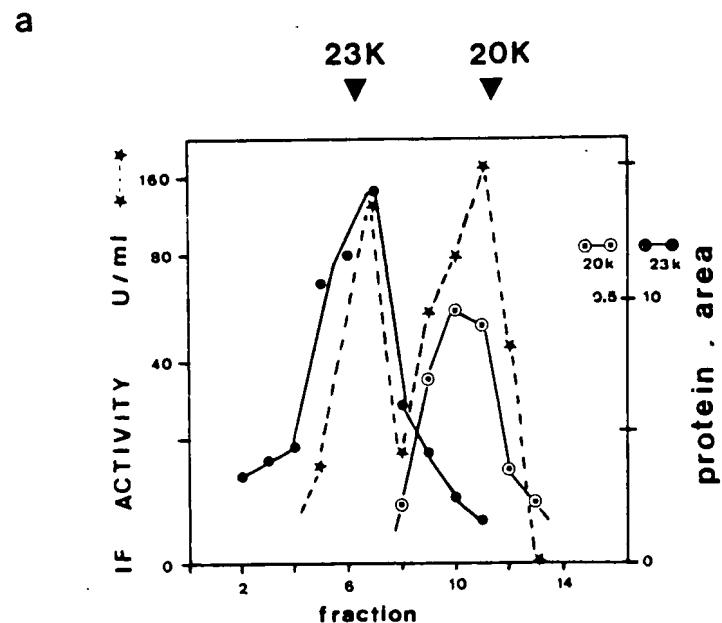
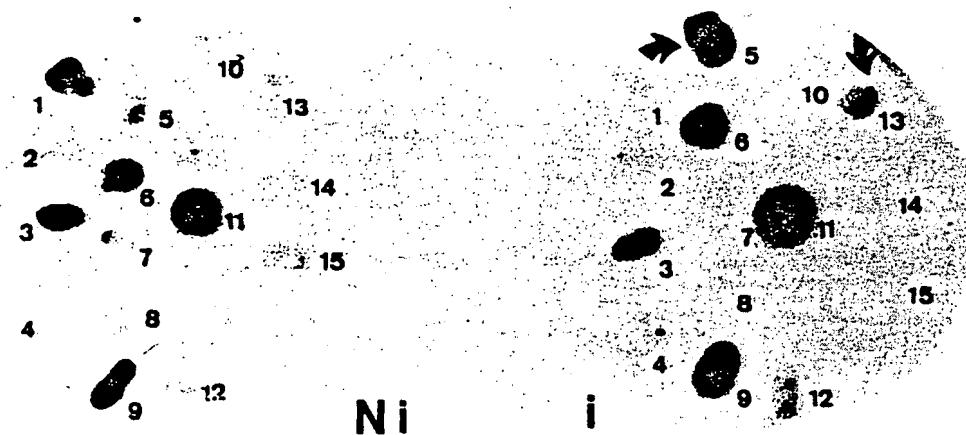


FIGURE 1



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FIGURE 2

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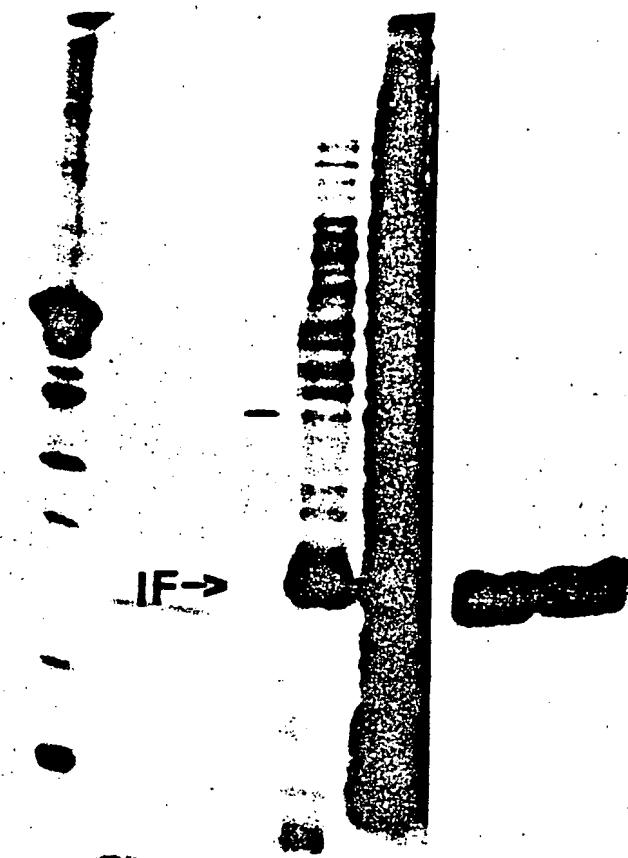


FIGURE 3

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a

1      2      3

i    n      i    n      i    n

-28s

-18s

-5s

A 341

pBR

tot.

b

DNA

B2

B1

1.3 kb —

0.9 kb —

FIGURE 4

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## SPECIFICATION

## Production of interferon

5 Interferon is an important antiviral and antitumor protein produced by the body. Because of its species specificity, clinical use of interferon requires human interferon. The limited amount of interferon that can be produced from tissue culture cells or from fresh white blood cells, is not sufficient for large-scale clinical use. Introduction of the genetic information for human interferon into a bacterial microorganism could possibly, if it were available, allow the mass-production of a polypeptide having interferon activity. It is known that

10 such techniques have been developed for human growth hormone and insulin.

The two types of interferon, i.e. leucocytes (IFN- $\alpha$ ) and fibroblast types (IFN- $\beta$ ) appear to be coded by different messenger RNAs (Cavallieri *et al.*, 1977, Proc. Natl. Acad. Sci. USA 74, 4415-19). Isolation of these mRNAs in pure form has not been achieved as yet. This appears to be all the more difficult as the host cell is liable of synthesizing the interferon mRNA only as a result of exposure of the latter to suitable exogenous factors, for instance, viral infection or particular experimental polynucleotides such as poly (rl:rC), and only in minute amounts. Accordingly, if it had already been suggested to use a mRNA extract of host cells which had preliminarily been induced to produce interferon and to cause it to be translated *in vitro* in a cell-free system comprising all ingredients, particularly the natural aminoacids, whereby a protein preparation having interferon activity had been obtained, the interferon mRNA was indeed but a very minor proportion 15 of the mRNAs being translated and accordingly the final protein preparation having interferon activity was 20 more than highly contaminated with other proteins.

Therefore, the use of the mRNA preparations of the prior art as a starting material for direct transforming into double stranded DNA liable of being cloned after its insertion in a suitable vector, would involve screening difficulties which would be insuperable in practice.

25 A major object of the present invention is to overcome to the greatest extent the above difficulties, particularly of providing a method of isolating the mRNA translatable into interferon, more particularly of human origin as well as the corresponding DNAs.

It is a further object of the present invention to provide a process for the isolation of genetic material (DNA) containing the nucleotide sequences coding for interferon in human cells.

30 It is yet a further object of the present invention to provide a process of this type wherein the messenger RNA is transcribed into DNA and which comprises cloning the DNA in a suitable vector. A preferred vector is a plasmid, and a preferred plasmid is a plasmid of the pBR322 type.

Furthermore it is an object of the present invention to provide a process to detect the different genetic sequence expressed in human cells when it is induced to produce interferon. A preferred embodiment

35 comprises effecting a differential hybridization of the bacterial clones first with DNA from induced-cells RNA and second with identical DNA derived from uninduced-cell RNA.

It is yet a further object of the present invention to provide a process of engineering a bacterial strain to produce interferon polypeptide which comprises introducing a cloned interferon DNA into a suitable vector-carrier. The cloned interferon DNA is advantageously produced as set out above. A preferred vector is

40 *E. coli* or another suitable microorganism. Another type of preferred vector-carrier is a eukaryotic cell.

Other and further objects of the present invention will become apparent hereinafter.

A preferred aspect of the present invention comprises a process to isolate genetic material (DNA) containing the nucleotide sequence coding for interferon in human cells, preferably fibroblastic cells, which comprises cultivating cells producing interferon when exposed to an inducer of interferon, exposing same to 45 such inducer, extracting messenger RNA from said induced cells, purifying the interferon messenger RNA, transcribing the messenger RNA into DNA and cloning the DNA in a suitable vector. According to a preferred embodiment the cells are human diploid foreskin cells. According to a preferred embodiment the inducer is double-stranded RNA.

The process according to the invention of enrichment of interferon mRNA or of a mRNA (inducible mRNA) 50 of other protein or polypeptide the production of which is liable of being induced in the host cell by exogenous factors comprises:

a) exposing a culture of the host cell to such exogenous factor to induce in said host cells the synthesis of the said inducible mRNA;

b) extracting the mRNAs including the inducible mRNA formed in said induced cell culture therefrom as

55 well as the mRNAs of a non-induced control culture of the same host cells;

c) synthesizing cDNA probes of the mRNAs of *both* the induced culture and the non-induced control culture using the corresponding mRNAs as templates (induced cDNAs and non-induced cDNAs);

d) synthesizing double-stranded cDNAs derived from the mRNA extracted from the induced culture, inserting said cDNAs in appropriate vectors, transfecting suitable microorganisms with the modified vectors

60 obtained and cultivating said microorganisms under conditions suitable to cause selective development of microorganisms colonies of said modified vectors (initial colonies);

e) forming duplicate colonies of said initial colonies;

f) causing freeing *in situ* of the DNAs of both said initial and duplicate colonies;

65 g) hybridizing the DNAs of the initial colonies on the one hand and the duplicate colonies on the other hand respectively with the abovesaid induced cDNA probes and non-induced cDNA probes (or conversely);

h) recovering DNAs of clones which hybridize with the induced cDNA probes and do not hybridize with the non-induced cDNA probes, whereby DNAs hybridizable with the mRNA capable of being translated into said inducible protein or polypeptide, particularly interferon, are obtained.

The process of the invention is preferably applied to the production of highly-purified mRNA of interferon 5 of human origin.

It will be appreciated that some of the steps defined above need not be performed in the exact order which has been indicated hereabove. This applies particularly to step c) relating to the synthesis of the cDNA probes. In fact the latter step c) even needs not be a step of the process of the invention *per se*. Analogous probes, from other cellular sources may be used instead.

10 In a further advantageous embodiment of the invention, steps c) and d), as above defined, are carried out 10 on fractions only of the mRNAs which can be extracted from cells, whether they have been "induced" or "non-induced". In that respect advantage can be taken of the fact that mRNAs comprise a poly-A portion which enables the separation of the mRNA from total RNA by binding to oligo-T-cellulose. The subsequently eluted mRNA fraction is advantageously subsequently fractionated by sucrose-gradient centrifugation, the 15 different bands being then separately translated into suitable cell-free system, such as a reticulocyte lysate, 15 each of the translation products being then tested for their capability of being immunoprecipitated by an anti-interferon serum. The bands of mRNA whose translation products gave a positive reaction in such immunoprecipitation test were then retained for carrying out the two above defined steps c) and d). A further aspect of this procedure is that two different mRNAs coding for interferon can be isolated from human

20 fibroblastic cells if these cells are induced as in a) above. The smallest mRNA sediments at 11S and yields by 20 translation in a cell-free system of protein of molecular weight 20,000 which is selectively precipitated by antibodies prepared against one of the interferons that can be purified from these cells. This protein is human interferon (IFN)- $\beta$ 1. The largest mRNA sediments at 14S and yields a protein of molecular weight 23,000 which is precipitated by antibodies against a less purified preparation of fibroblastic interferon. This 25 protein is designated human interferon- $\beta$ 2. Fractions of mRNA for both proteins were used for step d) above. 25 This makes possible to produce at will IFN- $\beta$ 1 or IFN- $\beta$ 2 in substantially pure form. Hybridization (steps c, d, e, f, g, and h defined hereabove) thus enabled single colonies of *E. coli* containing one of two interferon cDNA IFN  $\beta$ 1 or  $\beta$ 2 to be exactly identified. Particularly, it will be appreciated that those colonies which hybridize only with "induced" cDNA probes and not with the "non-induced" cDNA probes can only consist 30 of those which have incorporated a modified vector having the cDNA corresponding to the interferon mRNA, 30 inasmuch as:

a) the said DNA, not being hybridizable with the "non-induced" cDNA probe, does not accordingly correspond to any of the mRNAs normally produced by a "non-induced" cell and b) the only difference between the two probes being used is that the "induced" cDNA probe differs only from the other one by the 35 fact that it contains a cDNA derived from one of the interferon mRNA. It will be obvious to the man skilled in the art that the forming of these cDNA probes can be achieved by any method known *per se*, particularly by transcription in the presence of a reverse transcriptase. It is of advantage to use a high producer bacterial vector such as the pBR322 plasmid. In order to obtain the later expression in a microorganism of the vectors modified by the interferon cDNA, one may resort to a set of vectors as defined by Patrick Charney *et al.*, in 40 "Bacteriophage Lambda and Plasmid Vectors Allowing Fusion of Cloned Genes in each of the three 40 translational phases". Nucleic Acids Res., 1978 - 5(12), 4 479-94.

According to a further important step of the invention, the above modified vector, whichever be its translational phase, can be used for extracting interferon mRNA from the RNA mixture produced by "induced" cells, which process comprises contacting these RNAs with such a vector modified by the 45 interferon cDNA previously immobilized on a support, under conditions suitable for causing hybridization and thereafter eluting the fixed mRNA from the immobilized DNA vector. In such a way, mRNA of human interferon (either IFN  $\beta$ 1 or IFN  $\beta$ 2) may be obtained in highly purified form.

The high state of purification can be appreciated by the fact that the translation product, in a suitable *in vitro* system, consists in each case essentially of a single poly-peptide compound having interferon activity, 50 and which can be precipitated by said antibodies to human interferon.

The invention thus concerns also said purified mRNAs which comprises normally up to 900-1,000 nucleotides for IFN- $\beta$ 1 and 1,250-1,350 for IFN- $\beta$ 2. In the same manner it also concerns the corresponding cDNA which can be obtained by transcription of said RNAs. The invention concerns also the existence of two different interferon mRNAs and hence protein moieties that may have also different biological importance. It 55 goes without saying that each of the hybridization steps involved in the present application will be preceded, where appropriate, by a denaturation of the possible double-stranded nucleic acids to insure that no double-stranded nucleic acids (which could induce interferon activity in cells) remains in the purified mRNAs when these are used for the production, by translation thereof, of substantially pure protein having interferon activity *in vitro*.

60 The invention will be further illustrated in a non-limitative manner by the following more detailed description of a preferred embodiment thereof, in which reference is made to the Table given hereinafter and the accompanying drawings.

In the drawings:

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Figure 1 illustrates the fractionation of mRNA on a sucrose gradient and the translation of these mRNA 65 fractions originating from induced cells to produce human interferon activity in *Xenopus laevis* oocytes and

to produce specifically immuno-precipitated proteins in reticulocyte lysates, a process which separates the mRNA for IFN- $\beta$ 1 and IFN- $\beta$ 2.

Figure 2 illustrates the result of a differential hybridization procedure of the DNAs of the same bacterial colonies with the two above-mentioned probes from "induced" and "non-induced" cells, respectively.

5 5 *Figure 3 illustrates a purification of interferon IFN- $\beta$ 2 mRNA by hybridization to immobilized DNA from bacterial clone A341 as demonstrated by translation in a reticulocyte lysate, and*

5 *Figure 4 demonstrates that DNA from bacterial clone A341 is complementary to a 1,250-1,350 long mRNA which appears in human cells only after induction of interferon synthesis.*

10 *Table 1 demonstrates that this mRNA, upon translation in *Xenopus laevis* oocytes, yields biologically active interferon which inhibits the growth of a virus in human cells.*

a) *Purification of two interferon mRNAs from human diploid fibroblasts*

15 RNA was extracted from monolayer cultures of the human fibroblast line FS11 (isolated at the Weizmann Institute of Science). These diploid cells grown from foreskin explants taken from a normal individual 8 days after birth, were selected among 15 separate isolates for their capacity to produce high titers of interferon. Alternatively cultures of a clone of human SV80 cells were used. The cultures in Eagle's minimal medium with 10% fetal calf serum were maintained in 2.2 liter glass roller bottles or 22 x 22 cm plastic trays in 5% CO<sub>2</sub>, 95% air at 37°C. Three days after confluence, the cultures were induced to produce interferon by exposure to poly(rI):(rC) 100 µg/ml, and cycloheximide (which blocks the synthesis of proteins by the host) 20 50 µg/ml for 3.5 hours. Actinomycin D (which blocks the synthesis of cellular RNA) 1 µg/ml, was added and 1 hour later the cells were lysed with buffered Nonidet-P40 detergent and cytoplasmic RNA was extracted with a phenolcresol mixture as Kirby (1965). The mRNAs were isolated from total RNA, by bringing into play the fact that they contain Poly A, by binding to oligo-dT-cellulose. The mRNA fraction was subsequently fractionated by sucrose gradient centrifugation. The fractions containing interferon mRNA were identified by 25 microinjection to *Xenopus laevis* oocytes according to Raj N.K.B. and Pitha P.M. (Proc.Natl.Acad.Sci. USA 74, 1483-1487, 1977), and measuring 24-40 hours later the antiviral activity of the interferon released in the oocyte incubation medium. Antiviral activity was measured by exposing FS11 cells to dilutions of the oocyte medium, infecting said cells with vesicular stomatitis virus and observing inhibition of the cytopathic effect caused by the virus. Interferon titers were calculated by comparison to a known solution, according to the 30 last effective dilution. The fractions containing interferon mRNA were also identified by translation in a reticulocyte lysate followed by immunoprecipitation of the product according to the method of Weissenbach et al., (Eur. J.Biochemistry 98, 1-8, 1979).

35 Figure 1b shows the two peaks of interferon mRNA activity detected by injection to oocytes. Figure 1b is representative of the immunoprecipitation lines obtained between the translation products and antiinterferon serum, the two arrows showing the two polypeptides of molecular weight of 23,000 (23K) and 20,000 (20K). The sucrose gradient fractions coding for the 23K and 20K immuno-precipitated polypeptides are shown in Figure 1a and can be seen to correspond to the two peaks of interferon mRNA activity. Interferon activity was also detected in the translation products of reticulocyte lysates by measuring induction of the (2'-5') oligo-isoadenylate synthetase in human cells. By both methods it was seen that the largest interferon 40 mRNA peak codes for the 23K polypeptide, while the smallest interferon mRNA peak codes for the 20K polypeptide. Both interferon mRNAs were in this way isolated and used for cloning in *E. coli*.

b) *Cloning of interferon  $\beta$ 2 cDNA in *E. coli**

45 The purified mRNA from induced cells was calculated to contain about 1-3% of the mRNA for the 23,000 MW polypeptide and was used as template to synthesize cDNA with avian myeloblastosis virus, reverse transcriptase and oligo-dT as primer. After eliminating the RNA by alkali treatment, the second strand of DNA could be synthesized with reverse transcriptase or DNA polymerase I. Single-stranded DNA was hydrolyzed off with nuclease S1, and the 3' ends of the DNA was elongated ("tailed") with nucleotide terminal transferase using dGTP as substrate. The plasmid DNA was then hybridized with the dC-tailed 50 human cDNA described above, and used to transfet *E. coli* DP50. Transfected bacterial colonies were identified by plating on agar plate containing Luria broth, diaminopimelic acid, thymidine and tetracycline. The colonies were further tested on similar agar plates but containing ampicillin as the only antibiotic. The ampicillin sensitive, tetracycline resistant bacterial colonies were grown on a nitrocellulose filter deposited on an agar plate as above with tetracycline 10 µg/ml. Over two thousands of the transformed colonies 55 obtained were respectively transferred in part on other nitrocellulose filters, themselves on agar plates as hereabove indicated, each of the duplicate colonies being related (particularly by common numbering) to one of the initial colonies. After the colonies reached 3-5 mm in diameter, the filter (initial cultures and duplicates) were transferred on top of a stack of filter papers impregnated first with 0.5 N NaOH, then with 0.15 M NaCl and 0.1 N NaOH to cause release *in situ* of their respective DNAs. The filter were neutralized and 60 dried. To detect the bacterial colonies containing the interferon DNA sequences, the filters were hybridized with two different [<sup>32</sup>P]cDNA probes. One cDNA probe was prepared by reverse transcriptase of the mRNA from the sucrose gradient fraction from induced cells (arrow 23K of Figure 1). The second probe was prepared identically from the similar fraction of the non-induced cell preparation. Both cDNA probes were synthesized using the four highly radioactive [<sup>32</sup>P]-deoxynucleoside triphosphates as substrates and 65 fragmented calf thymus DNA as primers. Random representation of the mRNA sequences in the cDNA

probes was thereby achieved. Hybridization was carried out at 62-64°C for 18 hours in 0.9 M NaCl-0.09 M Na citrate buffer pH 7.0, the initial colonies being hybridized with the cDNA probes of the induced cells and the duplicate colonies with the cDNA probes of the non-induced cells (or conversely) respectively. After extensive washing the filters were exposed to X-ray film and the bacterial colonies able to hybridize to the induced cDNA but not to non-induced cDNA were identified. In this manner 20 different bacterial colonies were isolated out of a total of over 2,000 transformed colonies screened. All of these 20 bacterial colonies contain multiple copies of a plasmid in which were inserted sequences of human mRNA expressed only after cells have been induced to produce interferon by poly (rl;rC). 5

An example illustrating this technique is shown in Figure 2 in connection with fifteen pairs of alkali-treated pairs of colonies (initials and duplicates) on their nitrocellulose filters, whose DNA have been hybridized with  $[^{32}\text{P}]$ -cDNA prepared against mRNA fraction 23K of Figure 1, from cells induced (i) or non-induced (n.i.) by poly(rl):(rC) for interferon production. Arrows show two colonies, particularly colonies numbered 5 and 13, which contain induced sequences. Colony number 13 was designated as *E. coli* DP50/A341. 10

15 c) *Isolation of interferon mRNA from human fibroblast* 15

Isolation of interferon mRNA (and demonstration of the presence of interferon cDNA sequences in the plasmid DNA of clone A341) were obtained as follows: A 500 ml culture of this bacterial clone was used to prepare 50  $\mu\text{g}$  plasmid DNA. This DNA (after previous denaturation) was covalently bound to diazobenzyloxymethyl cellulose powder according to the methods of Aldwine *et al.* (Proc. Natl. Acad. Sci. USA 1977, 74, 5350). In parallel, plasmid pBR322 DNA (not containing human DNA sequences) was similarly bound to cellulose. Poly A-containing mRNA, from human fibroblasts induced to produce interferon, was hybridized to the two DNA cellulose preparations in 50% formamide at 52°C and eluted by raising the formamide concentration to 100% at 70°C. The RNA recovered after elution was translated in the reticulocyte cell-free system (Figure 3), whereby the essential translation product of the mRNA selected on the A341 20 DNA-cellulose was found to be essentially the 23,000 MW polypeptide. In contrast, no human interferon mRNA was recovered from the pBR322 DNA-cellulose. In comparison to the translation products of the human mRNA prior to hybridization to A341 DNA-cellulose it could be ascertained that the cloned A341 DNA is complementary to only little of the mRNA of the mixture. The product of the mRNA selected on A341 DNA-cellulose was immunoprecipitated by the anti-human fibroblast interferon serum. 25

25 The interferon mRNA could also be isolated by a similar procedure to that above but in which plasmid A341 DNA was bound to nitrocellulose filters, the RNA hybridized to it, and eluted by boiling for 1 min in  $\text{H}_2\text{O}$ . 30

30 The activity of this purified mRNA to code for biologically potent human interferon has shown by injection to *Xenopus laevis* oocyte followed by measuring the inhibition of virus multiplication in human cells exposed to the oocyte incubation medium (Table 1). The interferon activity of the purified  $\beta 2$  mRNA was also shown by the induction of (2'-5') oligo-isoadenylate synthetase in human cells by the oocyte translation products (Table 1). 35

35 Restriction enzyme mapping of A341 plasmid DNA showed that it contains a human DNA insert of about 900 nucleotides in the *Pst* site. The A341 DNA also hybridized to 3 fragments of the human genome digested by *Eco R1* nuclease. These fragments are separated by agarose gel electrophoresis. Hybridization to agarose gel electrophoregrams of mRNA from human fibroblast further showed that A341 DNA is complementary to RNA sequences that are expressed only in cells exposed to the interferon inducer poly (rl:rC) (Figure 4a). Even a one hour exposure of the cells to poly(rl:rC) leads to the accumulation of a 1,250-1,350 nucleotide long RNA hybridizing to A341 DNA, which represents IFN- $\beta 2$  mRNA. 40

40 45 The above data demonstrated that the bacterial clone *E. coli* DP50/A341 contained in the *Pst* site of its pBR322 plasmid an insert of about 900 nucleotides of human cDNA sequences which are complementary to a human interferon mRNA. Several similarly prepared clones were obtained. Figure 4b shows that clones for IFN- $\beta 2$  hybridize to the largest 1,250-1,350 nucleotide long mRNA while clones for IFN- $\beta 1$  hybridized to the smallest 900-1,000 nucleotide long mRNA. 45

50 50 The process can be used for obtaining clones of interferon DNA of different types ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) from human cells. 50

*Legends for figures:*

55 *Figure 1:* Sucrose gradient of poly A<sup>+</sup> RNA from human cells induced to produce interferon (a). Sedimentation was from right to left. Ten *Xenopus oocytes* were injected with 0.4  $\mu\text{g}$  RNA of each fraction and after 40 hours, the medium around the oocytes was assayed on FS11 cells for interferon (left scale). Each RNA fraction (0.24  $\mu\text{g}$ ) was translated also in reticulocyte lysates and the  $^{35}\text{S}$ -methionine labeled products were precipitated with anti-interferon serum. The products analyzed by polyacrylamide gel electrophoresis are shown in lane i of (b). Lane n in (b) represents the immunoprecipitated products of unfractionated mRNA from non-induced cells. At the right end of (b) are molecular weight markers (from top to bottom, 68, 46, 30, 18 and 14 daltons  $\times 10^{-3}$ ). The position and intensity of the 23K and 20K protein (arrows) was recorded and is shown graphically in a (right scale). The heaviest of the two interferon mRNAs is translated in the 23K protein while the smallest interferon mRNA is translated in the 20K protein. 55

60 *Figure 2:* Detection of transformed bacterial clone containing interferon DNA. Fifteen alkali-treated colonies on nitrocellulose filters, were hybridized with  $[^{32}\text{P}]$ cDNA prepared against the 23K mRNA fraction 60

(see Figure 1) from cells induced (i) or non-induced (n.i.) by poly(rI):(rC) for interferon production. Arrows show two colonies which contain induced sequences. Colony number 13 is *E. coli* DP50/A341.

Figure 3: Demonstration that clone *E. coli* DP50/A341 contains interferon DNA. Poly A<sup>+</sup> mRNA from human fibroblast induced for interferon production was hybridized to DNA from A341 plasmid covalently bound to cellulose and translated. Gel electrophoresis of the translation products show that mRNA which codes for the interferon (IF) polypeptide is uniquely selected from the mixture of total mRNAs. pBR DNA is unable to select this mRNA. Position of IF polypeptide is shown after immuno-precipitation with anti-interferon (ipt).

Figure 4: a) Plasmid DNA of bacterial clone A341 hybridizes to a 14S (1,300 nucleotides long) mRNA found in cells induced for interferon production (i) but not in non-induced cells (n). Plasmid pBR DNA does not hybridize while uncloned total cDNA (tot) hybridizes to many mRNAs found also in non-induced cells. An agarose gel electrophoresis of the RNA followed by hybridization to the three <sup>32</sup>P-DNAs is shown.

b) Plasmid DNA from different clones of interferon DNA were used for a similar experiment of hybridization to mRNA electrophoregrams. Clones containing IFN- $\beta$ 2 DNA hybridize to the 1,300 nucleotide long mRNA, while clones with IFN- $\beta$ 1 DNA hybridize to the smaller 900 nucleotides long interferon mRNA.

TABLE

Hybridization-translation of  $\beta$ 2-Interferon mRNA

	Expt. 1	Expt. 2	Calculated IF titer
	V.S.V.-Virus Yield Radioimmunoassay, cpm.	Oligo-isoadenylate synthetase induction [ <sup>32</sup> P]-A2'p5'A, cpm	
25	Oocyte supernatant (diluted 1:10)	Oocyte extract (diluted 1:15)	
30	- uninjected	7445	1700
	- with RNA hybridized to IF- $\beta$ 2 plasmid	1920	4700
35	- with RNA hybridized to unrelated plasmid	6015	1500
	Interferon standard 100 U/ml	700	10,800

\*Clone E474 DNA was used in Expt. 1, and a pool of IF- $\beta$ 2 DNA plasmids for Expt. 2.

## CLAIMS

1. A process to isolate genetic material (DNA) containing the nucleotide sequence coding for interferon in human cells which comprises cultivating cells producing interferon when exposed to an inducer of interferon, exposing same to such inducer, extracting messenger RNA from said induced cells, purifying the interferon messenger RNA, transcribing the messenger RNA into DNA and cloning the DNA in a suitable vector.
2. A process according to Claim 1, wherein the human cells are human diploid foreskin cells i.e. fibroblastic cells.
3. A process according to Claim 1 or 2, wherein the inducer is double-stranded RNA.
4. A process according to any of Claims 1 to 3 wherein the purification of the DNA is effected by sucrose gradient centrifugation following the *in vitro* translation of the messenger RNA into active interferon.
5. A process according to Claim 4 wherein the RNA is separated into RNA resulting in the ultimate production of human interferon IFN- $\beta$ 1 and in IFN- $\beta$ 2.
6. A process according to any of Claims 1 to 5 wherein the vector is a plasmid.
7. A process according to Claim 6 wherein the plasmid is pBR322.
8. A process to detect the different genetic sequence expressed in a human cell when it is induced to produce interferon which comprises effecting a differential hybridization of the bacterial clones first with DNA from induced-cells RNA according to Claim 3 or 4 and second with identical DNA derived from uninduced-cell RNA.
9. A process for engineering a bacterial strain to produce interferon polypeptide which comprises introducing a cloned interferon DNA obtained according to any of Claims 1 to 6, into a suitable vector-carrier.
10. A process according to Claim 9, wherein the vector-carrier is *E. coli*.

11. A process according to Claim 9, wherein the vector-carrier is an eukaryotic cell.

12. A process of enrichment of interferon mRNA or of a mRNA (inducible mRNA) of another protein or polypeptide the production of which is liable of being induced in the host cell by exogenous factors which comprises:

5    a) exposing a culture of the host cell to such exogenous factor to induce in said host cells the synthesis of the said inducible mRNA;

      b) extracting the mRNAs including the inducible mRNA formed in said induced cell culture therefrom as well as the mRNAs of a non-induced control culture of the same host cells;

      c) synthesizing cDNA probes of the mRNAs of *both* the induced culture and the non-induced control 10 culture using the corresponding mRNAs as templates (induced cDNA and non-induced cDNAs);

      d) synthesizing double-stranded cDNAs derived from the mRNA extracted from the induced culture, inserting said cDNAs in appropriate vectors, transfecting suitable microorganisms with the modified vectors obtained and cultivating said microorganisms under conditions suitable to cause selective development of microorganism colonies of said modified vectors (initial colonies);

15    e) forming duplicate colonies of said initial colonies;

      f) causing freeing *in situ* of the DNAs of both said initial and duplicate colonies.

      g) hybridizing the DNAs of the initial colonies on the one hand, and the duplicate colonies on the other hand respectively with the abovesaid induced cDNA probes and non-induced cDNA probes (or conversely).

20    h) recovering DNAs of clones which hybridize with the induced cDNA probes and do not hybridize with the "non-induced" cDNA probes, whereby DNAs hybridizable with the mRNA capable of being translated into said inducible protein or polypeptide, particularly interferon, are obtained.

13. The process of claim 12, wherein the starting mRNA of step a) of Claim 12 are of human origin.

14. The process which comprises introducing the DNAs of Claim 12 or 13 recoverable in the step h) of the 25 process of Claim 12 into a microorganism to form microorganism cells containing multiple copies of said 25 DNAs.

15. The vector which contains a DNA hybridizable with the RNA of an inducible protein, such as interferon.

16. The vector of Claim 15 which is derived of pBR322 and which contains a DNA insert hybridizable with 30 human mRNA and comprising up to 900-1,000 nucleotides.

17. The vector of Claim 15 or 16 whose DNA insert comprises about 720 nucleotides.

18. The process for separating the mRNA corresponding to an inducible protein, such as interferon, from the mRNAs produced by a cell, particularly of human origin, when the said cell is exposed to suitable 35 exogenous factor, which comprises contacting the previously extracted mRNAs formed by said cell with the DNA vector of any of claims 15 to 17 immobilized on a support under conditions suitable for causing hybridization, and thereafter eluting the fixed mRNA from the immobilized DNA vector.

19. The mRNA of human interferon in highly purified form.

20. The mRNA of human interferon  $\beta$ 1 in highly purified form.

21. The mRNA of human interferon  $\beta$ 2 in highly purified form.

40    22. The mRNA of human interferon which has from about 900 to about 1,000 nucleotides for IFN- $\beta$ 1 and 1,250-1,350 for IFN- $\beta$ 2 and which is translatable into substantially a single polypeptide having a molecular weight of about 20,000 or 23,000, respectively, interferon activity.

23. The DNA coding for a polypeptide having interferon activity insertable in a vector, such as plasmid pBR322.

45    24. Human interferon  $\beta$ 1 in highly purified form.

      25. Human interferon  $\beta$ 2 in highly purified form.

      26. A process for the production of interferon, substantially as hereinbefore described.

      27. Interferon when produced by a process according to claim 26.

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often as mesophilic cultures. Most infections found in our lab were caused by spore-forming species. This experience is also shared by Zeikus who reported the overgrowth of *Thermus* by *Bacillus* in improperly sterilized media and even growth of clostridial species in purely autotrophic cultures of *Methanobacterium*<sup>3</sup>. T. D. Brock wrote in 1969 that 'the quality of aseptic techniques of people working with thermophiles deteriorates with time, sometimes with disappointing results'. High cultivation temperature *per se* must not be regarded as a patent solution for improper or non-aseptic working techniques.

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ENCLOSURE

## Enzyme engineering: applications and promise

William H. Rastetter

Enzyme research and development have been shaped by the tools and concepts available for the production and utilization of enzymes. A new phase of enzymology has begun as recombinant DNA technology has made it possible to produce large quantities of pure, naturally occurring enzymes and to design modified enzymes with biotechnological uses.

Recombinant DNA (rDNA) technology provides a means of producing natural or modified proteins with a facility unprecedented in conventional protein chemistry. The design and rDNA-directed alteration of enzymes, or *enzyme engineering* offer a wealth of information on the relationship of protein structure to enzyme function and activity.

The structures of enzymes have evolved in response to the specific metabolic demands which these catalysts meet *in vivo*. Outside of the natural setting, however, an enzyme may not be well suited to an alternate use, e.g., as an industrial catalyst. Ideally and engineered enzyme will preserve or enhance the catalytic properties of the wild-type protein while bringing altered properties which are advantageous for industrial catalysts. For instance, improved thermal stability or an altered pH-activity profile would bring advantages to an enzymatic process stream which operates at non-physiological temperature and pH. Thus, enzyme engineering entails the

tailoring of protein catalysts to meet the specific demands of the end user.

More broadly, protein engineering may apply to all classes of protein products which emerge from the rDNA industry, including enzymes for therapeutic or diagnostic use, the interferons, growth hormones and a variety of other proteins of potential value in human and animal health care. Engineered proteins for pharmaceutical or veterinary use may display enhanced pharmacokinetics and altered activities, or increased stabilities as formulated products.

### Study of enzyme structure and function

To study enzyme structure and function systematically requires the means to make selected structural changes in the enzyme. Naturally occurring mutant enzymes provide a set of proteins for examination, but the number and variety of such proteins are limited. Moreover, the total chemical synthesis of enzymes and mutant enzymes from amino acids is impracticable since the chemical synthesis of even small proteins is extremely laborious. Synthetic studies have focused, instead, on the chemical modification of enzymes of natural

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origin and on the assembly of smaller molecules as models for enzyme activity. Relatively small molecules such as the crown ethers<sup>1</sup>, cryptands<sup>2</sup> and altered cyclodextrins<sup>3,4</sup> share some of the binding and catalytic properties of enzymes.

As a class, however, the enzymes are unequalled as versatile catalysts. Their size often allows the substrate to be enveloped intimately by the protein molecule. The tight interaction achieves specificity for substrate, stereoselective reactivity and, through tight transition state binding, rate acceleration. The infinite variety of amino acid sequences can yield subtle local variations in structure, providing the varied effects of hydrophobic and hydrophilic interactions, solvation, and binding pockets for substrate, cofactors and allosteric factors. The ability to build and study unnatural catalysts with such structural variation and complexity until recently has been limited, not by the imagination, but by the tools available to the chemist.

## Glossary

**enzyme engineering** – often used to mean the industrial utilization of enzymes, but here restricted to the design of protein catalysts, not overall industrial processes.

**fusion protein** – a protein consisting of all or part of the amino acid sequences of two or more proteins, e.g. *E. coli*  $\beta$ -galactosidase and human proinsulin. The regulatory sequence of a bacterial gene (e.g. promoter, ribosome-binding site) often must be attached to a foreign gene if the latter is to be expressed in the bacterial host. In many cases there is no known restriction enzyme that can cleave only the required regulatory sequences from the bacterial gene so that these alone can be linked to a foreign gene. Thus these sequences plus a portion (or all) of the coding sequence for a bacterial gene may be attached to the foreign gene which is to be cloned. Sometimes the bacterial protein fragment of the fusion protein plays a positive biotechnological role, for example, allowing the host to secrete the fusion protein.

**real-time** – the ability, at the graphics screen, to instantaneously and continuously rotate and translate the model, or portions of the model, in response to the operator's commands. The capability is made possible by very rapid computation of new atomic coordinates and consequent rapid changes in the graphics display which simulate the motion of the model.

## Enzyme engineering

The implications of rDNA methodology for the study of structural enzymology are far reaching. The chemical synthesis of oligonucleotides, coupled with the enzymatic methods of genetic engineering, allows the assembly and replication of altered genes and hence proteins of altered amino acid sequence. This opens the way to experimentation in the relationship of protein structure to enzyme function and activity which was not previously thought possible. Indeed, the opportunity offered by rDNA technology presents a dilemma to the enzymologist. Even for the small protein, utilization of rDNA-directed mutagenesis for the production of altered structures must be done with some guiding design principles. The number of amino acids in any enzyme is too large for us to consider making a comprehensive study of amino acid substitution.

X-ray crystallography, the essential tool in determining the three-dimensional shapes of proteins, provides a structural basis to guide selective enzyme alteration. From a crystalline protein, X-ray diffraction techniques may provide a map of electron densities in the macromolecule. In a high-resolution structure, features such as disulfides, aromatic rings and  $\alpha$ -helices are clearly discernible from the electron density patterns. For proteins of known amino acid sequence a molecular model representing bond lengths and angles can be fitted to the three-dimensional map of electron density. This model may be an assembly of colored wire, wood or plastic, but increasingly crystallographers have made use of computer graphics for model building. The model on the graphics screen (for reviews see Refs 6-9) is easily assembled, manipulated, stored and retrieved.

The graphics molecular model and time-honored chemical principles, developed initially from small molecule interactions and reactivities, form a good basis for selective enzyme modification. Graphics software provides much more than the wire, stick model (Dreiding model) or the plastic, space-filling model (Corey-Pauling-Koltun or CPK model). The computer will provide a representation of the backbone of the protein, with or without the amino acid side chains, as well as the solvent-contact surface or electrostatic surface of the macromolecule. The

graphics screen can depict – in color, in perspective, and in real-time – the translation and rotation of an enzyme and its substrate. The graphics tool affords the viewer any perspective from outside or inside of the enzyme, and allows the macromolecule to be viewed one slice or section at a time. Equipped with a stereoviewer, the graphics screen treats the operator to the illusion of depth in a graphic representation which, as a surrogate enzyme, becomes a powerful design tool.

The use of X-ray crystallography and molecular modeling in an interactive approach to enzyme engineering is depicted in Fig. 1. From a wild-type gene, the techniques of genetic engineering provide a plentiful source of the corresponding protein. The wild-type protein may be assayed to deter-

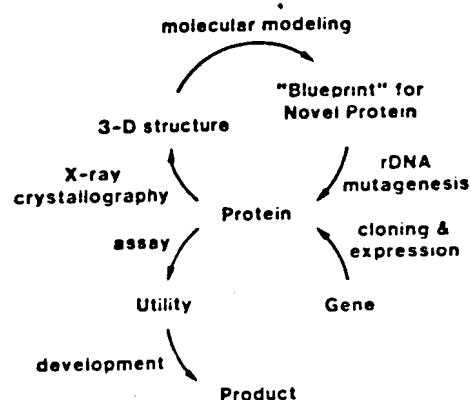


Fig. 1. The iterative steps of enzyme engineering.

mine its market potential, and in many cases, the wild-type protein will be directly developed into a product. By contrast, second generation products and enzymes for novel applications will be derived from the design loop which utilizes X-ray crystallography and molecular modeling. From the three-dimensional structure of an enzyme, a blueprint for an altered, unique enzyme can be obtained by applying the concepts of enzyme structure and mechanism of action. The novel structure, initially represented only on the computer graphics screen, can be tested through rDNA-directed mutagenesis of the wild-type gene and microbial biosynthesis and assay of the new protein.

The protein engineering process can, and should, be iterative. Crystallographic characterization of mutant proteins will provide feedback for the design process by revealing the actual three-dimensional structure for the altered protein. Subtle changes in struc-

are not predicted by the graphics model may be allowed for in a second iteration of the design loop. For easily assayed mutant proteins, the iterative steps of the design process can proceed rapidly. Both the production and the crystallographic characterization of mutant proteins should be facilitated by information and material from the wild-type system, and the production and characterization of families of mutant proteins should come rapidly once the parent systems are well understood.

### Some early engineering efforts

Examples of enzyme (protein) engineering have begun to appear in the scientific literature. Targets for rDNA-directed mutagenesis have been chosen among proteins with a known or putative relationship between structure and function. This approach has undoubtedly facilitated the interpretation of experimental results and has guided the experimentalist in the choice of site(s) for mutagenesis.

$\beta$ -Lactamase is a hydrolytic enzyme of bacterial origin (encoded by the ampicillin-resistance gene, ( $\text{Ap}^R$ ), of plasmid pBR322) which deactivates the  $\beta$ -lactam ring of penicillin derivatives. The enzyme is biosynthesized as a precursor protein with an amino-terminus extension or signal sequence of 23 amino acids. The signal sequence is required for the secretion of  $\beta$ -lactamase into the periplasmic space (between inner and outer membranes) of Gram-negative bacteria and is removed during the transport process. Directed mutations have provided preliminary information on structural features within the signal sequence<sup>10,11</sup> and the mature enzyme<sup>12</sup> which influence secretion and the removal of the signal sequence from the precursor protein. Such studies will undoubtedly increase our knowledge of the mechanisms of cellular secretion and lead to the design of fusion proteins engineered for efficient secretion during microbial fermentation. A more detailed examination of the effect of structure and charge in the signal sequence of the prolipoprotein from *E. coli* has been reported by Inouye and co-workers.<sup>13</sup>

The active site of  $\beta$ -lactamase has also attracted attention for structural studies by directed mutagenesis. Sigal *et al.* have reported<sup>14</sup> the replacement of the active site serine by cysteine,

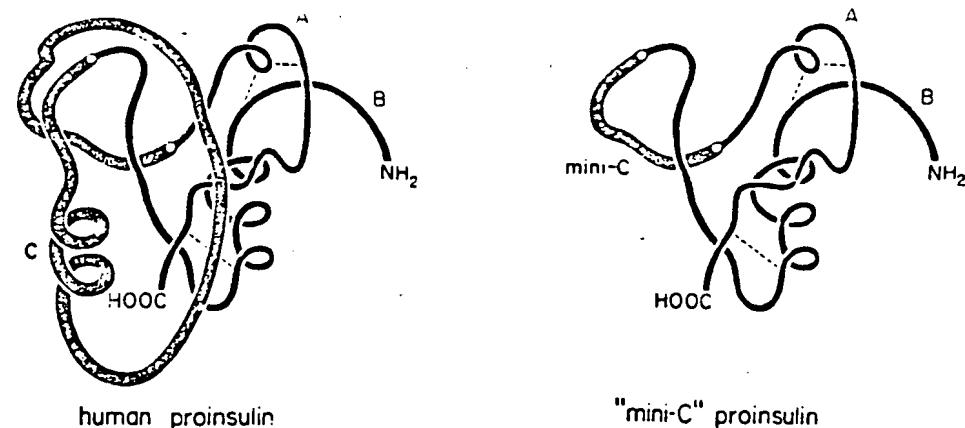


Fig. 2. Representations of native proinsulin and 'mini-C' proinsulin showing the shortened connecting peptide in the engineered protein. The conformations of the A- and B-chain segments are taken from X-ray crystallographic data; the conformations of the connecting peptides were arbitrarily selected for the illustration. The overall conformation for 'mini-C' insulin is assumed from the chromatographic and radioimmunoassay behavior of the refolded molecule (see text).

yielding a thiol- $\beta$ -lactamase with reduced activity *in vitro* and *in vivo*. The activity of the mutant enzyme is rapidly eliminated by the thiophile, *p*-chloromercuribenzoate.

Richards and co-workers<sup>15</sup> have completely removed the catalytic activity of  $\beta$ -lactamase in a mutant protein in which the amino acids at active site positions 70 and 71 (Ser-Thr) have been reversed (Thr-Ser). Catalytic activity may depend on the presence of a primary hydroxyl group in the side chain of amino acid 70 (as in Ser) rather than a bulkier, secondary hydroxyl group (as in Thr). Random mutagenesis of the inactive Thr-Ser mutant cloned in *E. coli* yielded bacteria which were resistant to ampicillin, indicating that a further mutation had converted the inactive enzyme to an active form. Sequencing of the gene subjected to the random mutagenesis revealed, not reversion to wild type (Ser-Thr), but a single base change in the codon for amino acid 70 giving an active enzyme with the amino acid dyad, Ser-Ser, at positions 70 and 71.

Insulin is assembled at the ribosome as a single polypeptide chain containing an amino-terminus signal peptide and an interstitial C-peptide. Maturation of insulin involves secretion and removal of the signal peptide, folding of the resultant proinsulin with formation of three disulfide bonds, and removal of the 35-amino acid C-peptide by the enzymatic cleavage of proinsulin at two sites. Removal of the C-peptide leaves the two separate polypeptide chains of insulin (A and B chains) connected covalently by disulfide bonds (see proinsulin in Fig. 2).

The X-ray crystallographic structure for insulin<sup>16,17</sup> shows that a peptide much shorter than the 35-amino acid C-peptide of human proinsulin could be made to span the gap between the carboxy-terminus of the B chain and the amino-terminus of the A chain (Fig. 2). Such a 'mini-C' analog of insulin produced by recombinant techniques is predicted to fold into a conformation closely resembling native proinsulin. Wetzel and co-workers<sup>18</sup> have constructed a gene coding for mini-C proinsulin in which six amino acids (Arg-Arg-Gly-Ser-Lys-Arg) replace the native C-peptide. The gene was cloned as a  $\beta$ -galactosidase fusion protein in *E. coli*, and the proinsulin analog obtained after cleavage by cyanogen bromide and purification. Folding of the analog was achieved under conditions favoring cysteine interchange and the formation of disulfide bonds in native proinsulin; this generated a molecule having chromatographic and radioimmunoassay behaviour consistent with a proinsulin-like structure.

Engineered proteins such as mini-C proinsulin may find applications as pharmaceutical products. For example, the pharmacokinetics of the processing *in vivo* of mini-C proinsulin to the active hormone, insulin, probably would differ from that of natural proinsulin. A controlled-release formulation of proinsulin for human administration, thus, might contain engineered sequences within the C-peptide. The possible antigenicity of such preparations of unnatural proteins would have to be evaluated.

The X-ray crystallographic structure of tyrosyl tRNA synthetase of *Bacillus*

*stearothermophilus* is known to  $\sim 3^{\circ}$  resolution and the binding sites 1 tyrosine and ATP have been identified. Winter and colleagues<sup>19</sup> have targeted cysteine 35 as a site for mutagenesis based on the contact of the cysteine side chain with the 3' hydroxyl of the sugar moiety of tyrosyl adenylate as seen in the X-ray crystal structure. Replacement of cysteine 35 by serine yields a mutant enzyme which is intrinsically less active than the wild type. The reduction in activity is attributed largely to an altered  $K_m$  for ATP.

The recent surge of papers on techniques for rDNA-directed mutagenesis attests to the rapid development and broad interest in the new methodology. An exhaustive review of the techniques for mutagenesis is beyond the scope of the present paper. A few leading papers and reviews should be noted, however.

Hutchison, Smith and colleagues have described<sup>20</sup> a method for changing a specific nucleotide in a DNA sequence with high efficiency. A synthetic oligonucleotide complementary to the wild-type sequence except for the altered nucleotide(s) is used as a primer for the enzyme, DNA polymerase, on a circular, single-stranded wild-type DNA template. Isolation of mutant sequence is achieved after biological replication, i.e., by cloning. The efficient correction of a mutation also by use of a chemically synthesized oligonucleotide as a primer for DNA polymerase was described by Razin *et al.*<sup>21</sup> Use of a chemically synthesized oligonucleotide to introduce a mutation and as a probe to screen transformed bacterial colonies for the desired mutant has been exploited by Wallace *et al.*<sup>22</sup> and by others<sup>23</sup>. Smith and Gillam have reviewed<sup>23</sup> the use of synthetic oligonucleotides as site-specific mutagens, and correctly suggest that potential applications of the method are legion. Bostein, Shortle and Rose also have reviewed<sup>24</sup> other techniques for mutagenesis of cloned genes.

As recent examples of protein engineering illustrate, rDNA techniques allow proteins to be modified by amino acid substitution at any position(s). The power of these techniques will greatly extend the limited studies done with proteins modified by chemical and/or enzymatic techniques. The direct modification of proteins relies on the selective transformation of complex macromolecules rather than the directed and predictable synth-

of gene segments involved in recombinant mutagenesis. The former approach can rarely give the homogeneous, modified protein which is routinely produced by gene-splicing techniques.

The early chemical and enzymatic, protein-modification experiments, nonetheless, are the conceptual forerunners of today's rDNA-directed protein engineering efforts. Two examples are noted here to illustrate the approach and the scope of direct protein modification.

The chemically modified enzyme, thiol-subtilisin<sup>25,26</sup>, was prepared by conversion of the active site serine of subtilisin into a cysteine residue (compare thiol- $\beta$ -lactamase, above). While specific chemical reactions at single sites within proteins are not always feasible, in subtilisin the unique reactivity of the active site serine allowed its selective modification. The serine-to-cysteine transformation was accomplished via activation of the serine residue with phenylmethane-sulfonyl fluoride, followed by reaction with thioacetate and deacylation of the product, acetylthiol-subtilisin. Thiol-subtilisin will hydrolyse labile *p*-nitrophenyl esters, but is inactive toward normal peptide and ester substrates.

By a similar strategy, Laskowski and co-workers<sup>27-29</sup> in early work exploited the unique reactivity of the arginine-63-isoleucine-64 bond of soybean trypsin inhibitor toward trypsin in the direct enzymatic modification of the protein inhibitor. Modification of the residues at the cleavage site (between residues 63 and 64) allowed the activity of the inhibitor to be modulated. While substitution of alanine at position 64 yielded a fully active trypsin inhibitor, substitution by tryptophan at position 63 gave an inhibitor for chymotrypsin. Insertion of glutamate at the cleavage site converted the trypsin inhibitor into a trypsin substrate. Other examples and a discussion of techniques for direct protein modification have been reviewed<sup>1</sup>.

Other reviews and comments on enzyme (protein) engineering have appeared in the literature. Ulmer<sup>30</sup> has reviewed the general approach and rationale for protein engineering, and Pabo<sup>31</sup> has discussed the general problem of protein and peptide design. On a more futuristic note, Drexler<sup>32</sup> has discussed protein engineering as an approach to the development of general

capabilities for molecular manipulation, i.e., the use of proteins as tools for the microtechnologist. Among Drexler's suggestions are the assembly of molecular scale units for the storage and rapid processing of information and the *de novo* design and assembly of complex molecular machines (artificial enzymes) to direct chemical synthesis.

### Prospects for enzyme engineering

The study of protein structure is no longer limited by the ability to obtain altered proteins. Site-specific mutagenesis allows proteins to be altered with precision. Mutations not easily achieved by classical techniques, e.g., contiguous point mutations, are easily achieved with the newer techniques. Hybrid proteins and even protein design *ab initio* is within the scope of the technology. The field will be limited only by the ability to select the proper amino acid sequences for engineered proteins. Yet, as only a small subset of possible mutant proteins can be examined in the design and production of engineered proteins, target proteins must be carefully considered and research guided by tools such as X-ray crystallography and molecular modeling.

Clearly, however, the predictive power of X-ray diffraction techniques and molecular modeling will be much greater for some amino acid substitutions than for others. Mechanism-based inactivation of enzymes will be easy to predict given a known mechanism of action for an enzyme. By contrast, changes which improve catalytic efficiency will be more difficult to predict, given the precise alignment of functional groups which probably is necessary for efficient catalysis.

In enzyme engineering, initial successes may come from modest changes which are not directly related to the mechanism of catalysis. For example, a model incorporating changes at the binding or allosteric sites of an enzyme may provide a catalyst with altered substrate specificity or sensitivity to product inhibition. Such rationally selected changes in protein structure, not selected for in the course of mutagenesis *in vivo* will yield enzymes with enhanced characteristics for industrial biocatalysis.

Changes in enzyme properties which may be difficult to achieve by a rational design process and site-specific mutagenesis may be achieved through other

mutagenesis techniques. Mutagenesis in continuous culture or in nature may provide active mutant enzymes, especially when the mutant provides a selective advantage to its host organism. Recombinant techniques also allow saturation mutagenesis of selected regions or sites of a gene. By these techniques, families of related enzymes are produced in a single cloning exercise. A population of bacterial colonies so produced will carry the mutant genes; each colony will harbor the gene coding for a single recombinant enzyme. Screening techniques may allow the colonies to be rapidly examined for mutant protein activity. Only the subset of colonies containing active enzymes will require further characterization. A comparison of mutant and wild-type proteins (or genes) will reveal sites which modulate activity or function. Such clues will greatly facilitate the rational, iterative design approach (see Fig. 1).

The diversity of function and range of properties of the natural enzymes reveal the broad scope of utility and variation which has evolved for the protein catalyst. The enzymologist, using the tools of rDNA technology complemented by screening and selection techniques, will build an increasingly diverse group of unique, man-made enzymes. The list of enzyme properties which will be targeted and enhanced or modulated for various applications is long. These properties include: substrate and cofactor specificity and affinity; reaction stereoselectivity; feedback inhibition; stability to non-aqueous solvents, heat and oxidizing media; stability *in vivo*; susceptibility to proteolysis; antigenicity; pH, ionic strength and temperature optima; catalytic efficiency; enzyme function and physical and chemical behavior during purification and/or immobilization or formulation. The group of novel enzymes produced by genetic engineering will grow, initially, as limited, selected changes are made in wild-type catalysts. As we grow confident in the design and assembly of protein structural units, hybrid enzymes will become available bringing function and properties from two or more natural, protein catalysts. Ultimately, when protein folding can be predicted by computational methods and proteins can be characterized more rapidly by newer crystallographic techniques, *de novo* protein design and

synthesis will be possible. Protein domains or subunits will be assembled and linked at the graphics screen in a computer assisted design approach to catalyst production. The design at the graphics screen will be tested, probably domain by domain, through gene synthesis and expression, and protein isolation and characterization. These rationally conceived and deliberately assembled proteins will provide the ultimate and rigorous test of our ability to understand and manipulate protein structure, function and activity.

How far off is *de novo* enzyme design and synthesis? Ten, fifteen years? Perhaps not that long. The answer depends largely on the development of more sophisticated knowledge of protein structure and the nature of enzyme catalysis, with an emphasis on *predictive* capability, and the development of more powerful and rapid methods for protein structural characterization. The recombinant DNA methodology for *de novo* protein synthesis exists today. A step at a time we will move away from the wild-type enzyme toward the totally synthetic protein catalyst. The new field of enzyme engineering is fertile for discovery.

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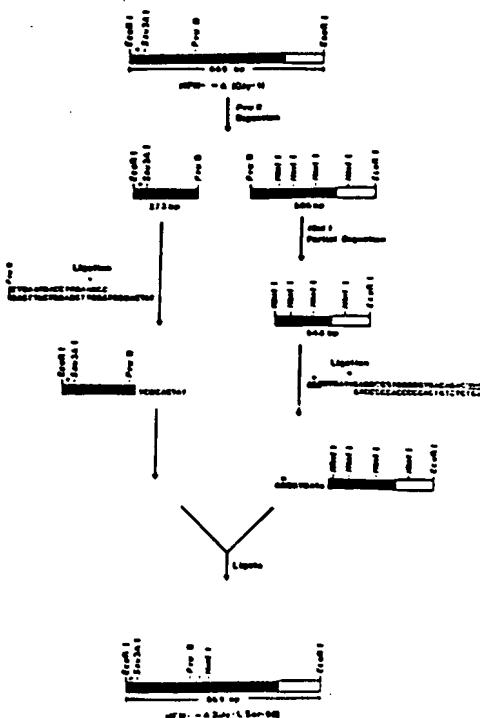
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#### 54 Polypeptides having Interferon activity.

57) Polypeptides having interferon activity with an amino acid sequence corresponding to the amino acid sequence of an interferon selected from a rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  in which at least one cysteine residue has been replaced by an amino acid residue which is incapable of forming an intermolecular disulfide bond; DNA sequences coding for these polypeptides; replicable plasmidic expression vehicles containing these DNA sequences; microorganisms transformed with these expression vehicles; and methods of preparing them.



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Polypeptides having Interferon Activity

5        Interferons are proteins which are synthesized by cells in response to viral infection, immune stimulation and a variety of inducers and which are presently divided into three major classes: alpha or leukocyte interferon (IFN- $\alpha$ ), beta or fibroblast interferon (IFN- $\beta$ ) and gamma or immune interferon (IFN- $\gamma$ ). The interferons have anti-viral and antiproliferative activities, a potent ability to confer a virus-resistant state in targeted cells and immunomodulatory activities (J.L. Toy, Clin. exp. Immunol. 54, 1-13 [1983]). Their biological properties have led to the 10      clinical use of interferons as therapeutic agents for the 15      treatment of viral infections and malignancies.

20        Interferons have been obtained from natural sources such as leukocytes derived from the buffy coats of whole blood, lymphoblastoid cells in continuous suspension culture or fibroblast cultures upon stimulation with inducing agents. Cells which can be stimulated to produce IFN- $\gamma$  include both B and T cells. The interferons obtained from these sources have been purified to homogeneity. Interferons have also been produced using methods of recombinant 25      DNA technology, i.e. by expression from microorganisms transformed with expression vectors containing interferon genes under the control of suitable promoter-operator sequences. The leukocyte, fibroblast and immune interferons produced by recombinant techniques are designated rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$ , respectively. Since at least 12 30      distinct IFN- $\alpha$  genes have been identified the corresponding proteins are designated rIFN- $\alpha$ A, rIFN- $\alpha$ B, rIFN- $\alpha$ C and so forth. The amino acid sequences of 35      rIFN- $\alpha$ A-L, as well as the nucleotide sequences which

encode them are described, e.g., by Pestka in Archiv. Biochem. Biophys. 221, 1 (1983) and in British Patent Specification No. 2 079 291.

5        Goeddel and coworkers achieved the initial expression of rIFN- $\alpha$ A in *E. coli* cells transformed with the recombinant plasmid pL 31 (Nature, 287, 411-416 [1980]). This plasmid contains the structural gene for mature rIFN- $\alpha$ A (i.e., a gene in which the nucleotide sequence encoding a  
10      23-amino acid signal peptide normally translated in the human cell has been removed and an ATG "start" signal has been inserted immediately before the codon for the first amino acid following the signal peptide) under the control of an appropriately positioned promoter-operator sequence.  
15      The rIFN- $\alpha$  interferons are 165 amino acids (in the case of rIFN- $\alpha$ A) or 166 amino acids in length, except that they may, in some instances contain an additional methionine attached to the N-terminus of the ordinarily first amino acid of the protein as the result of translation of  
20      the ATG start signal which encodes methionine.

25      Hybrid leukocyte interferons have been produced by expression of genes which are produced by cleaving two or more IFN- $\alpha$  genes at internal endonuclease cleavage sites and then ligating one or more fragments of one gene with one or more fragments of a different gene (or genes) to produce a new gene encoding a complete 165- or 166-amino acid IFN- $\alpha$  with specific segments corresponding to portions of different IFN- $\alpha$  species. In this manner, for  
30      example, it has been possible to produce a leukocyte interferon in which the amino acid sequence corresponds to amino acids 1-91 of rIFN- $\alpha$ A followed by amino acids 92-166 of rIFN- $\alpha$ D. Similarly, it has been possible to produce a hybrid IFN- $\alpha$  in which the amino acid sequence corresponds to amino acids 1-92 of rIFN- $\alpha$ D followed by amino acids  
35      93-165 of rIFN- $\alpha$ A (J. Biol. Chem. 257, 11497-11502 [1982]).

494

The cloning and expression of mature rIFN- $\beta$ , its amino acid structure, as well as the nucleotide sequence coding therefor are described by Goeddel et al. in Nucleic Acids Research 8, 4057 (1980).

5

The cloning and expression of mature rIFN- $\gamma$ , its amino acid structure and the nucleotide sequence coding therefor are described by Gray et al. in Nature 295, 503 (1982).

10

However, allelic variations of the specific amino acid sequences of the above-cited interferons are possible and have already been described. For example, Nagata and coworkers described the expression of an rIFN- $\alpha$  gene (denoted as rIFN- $\alpha_1$ ) which yielded a polypeptide (in non-mature form) differing from rIFN- $\alpha$ D by a single amino acid at position 114 (Nature 284, 316 [1980]). Similarly the cloning and expression of another rIFN- $\alpha$  gene (identified as rIFN- $\alpha_2$ ) which yielded a polypeptide differing from rIFN- $\alpha$ A by a single amino acid at position 23 has been described in European Patent Application No. 32 134, published July 15, 1981.

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A problem which has occurred in the manufacture and use of interferons is that the individual interferon molecules tend to oligomerize. While the etiology of these oligomers has not been completely understood it is believed that the methods used to purify interferons for therapeutic use such as high pressure liquid chromatography or monoclonal antibody affinity chromatography may contribute to the formation of dimers, trimers and higher oligomers of interferon. These oligomeric forms which have been observed particularly with respect to leukocyte and fibroblast interferons result from two or more interferon molecules becoming irreversibly associated with one another through intermolecular covalent bondings, such as disulfide linkages.

While the dimeric forms of interferons are believed to retain biological activity, the higher oligomeric forms in many cases have either no biological activity or significantly reduced activity by comparison to the monomeric forms. Moreover, the oligomeric forms have the potential for causing deleterious side effects if used therapeutically.

All of the known rIFN- $\alpha$ s, rIFN- $\beta$  and rIFN- $\gamma$  contain several cysteine residues the sulfhydryl side-chains of which are capable of forming intramolecular and intermolecular disulfide bonds, which latter result in oligomerization. The amino acid sequence of rIFN- $\alpha$ A contains cysteine residues at positions 1, 29, 98 and 138. Wetzel assigned intramolecular disulfide bonds between the cysteine residues at positions 1 and 98 and between the cysteine residues at positions 29 and 138 (Nature 289, 606 [1981]).

Heretofore, efforts to overcome the oligomerization problem have concentrated on adjusting the purification conditions in order to prevent the formation of oligomers or on post-processing reaction conditions whereby intermolecular disulfide bonds are reduced.

The present invention in a first broad aspect relates to novel polypeptides having interferon activity comprising an amino acid sequence corresponding to the amino acid sequence of an interferon selected from a rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  wherein, however, at least one cysteine residue has been replaced by another amino acid residue, i.e. an amino acid residue which is incapable of forming an intermolecular disulfide bond.

In a preferred embodiment of this broad aspect, the invention relates to a polypeptide with the amino acid sequence of rIFN- $\alpha$ A in which, however, at least one of

the cysteine residues at positions 1 and 98 has been replaced by another amino acid residue which is incapable of forming an intermolecular disulfide bond.

5 The invention also relates to pharmaceutical compositions with antiviral activity containing the novel polypeptides and which are essentially free of oligomers other than dimers and, preferably, contain only a stable monomeric interferon.

10 In a second broad aspect, this invention relates to methods and intermediates for producing the novel polypeptides described above by recombinant DNA technology. In particular, this aspect relates to a method of producing a double stranded DNA (dsDNA) encoding the novel polypeptides by using restriction enzymes to excise a portion of the dsDNA comprising the codon for the undesired cysteine residue from the parental interferon gene and replacing it with a synthetic oligodeoxynucleotide segment wherein the nucleotide triplet coding for cysteine has been replaced by a nucleotide triplet coding for another amino acid residue which is incapable of forming an intermolecular disulfide bond.

25 Accordingly, this second broad aspect of the invention encompasses dsDNA sequences coding for the novel polypeptides, replicable plasmidic expression vehicles containing these dsDNA sequences, microorganisms transformed with these replicable plasmidic expression vehicles and a method of preparing these dsDNA sequences.

30 Preferred polypeptides of the invention are those obtained by substitution of cysteine residues in rIFN- $\alpha$ s and hybrid rIFN- $\alpha$ s. All rIFN- $\alpha$ s and corresponding hybrid rIFN- $\alpha$ s contain a cysteine residue at the N-terminus, i.e. at position 1. Additionally, rIFN- $\alpha$ A contains cysteine residues at positions 29, 98 and 138, rIFN- $\alpha$ B at

positions 29, 100 and 139; rIFN- $\alpha$ C, F, G, H, I, J, K and L at positions 29, 99 and 139; and rIFN- $\alpha$ D at positions 29, 86, 99 and 139. It has to be noted that Pestka, in Archiv. Biochem. Biophys. 221, 1 (1983), Fig. 17, shows cysteine residues at positions 99 and 139 of rIFN- $\alpha$ A rather than at positions 98 and 138. This is because the sequence representation has been shifted one position following position 43 to align it with the other rIFN- $\alpha$  species which contain one more amino acid residue than rIFN- $\alpha$ A. All amino acid positions recited herein refer to sequentially numbered amino acids and do not account for such shifts.

It is understood that the polypeptides of the invention have amino acid sequences corresponding to amino acid sequences of known rIFNs wherein, however, at least one cysteine residue has been replaced by another amino acid residue which is incapable of forming intermolecular disulfide bonds. The cysteine residues which are replaced are those which are not required to maintain interferon activity of the molecule. As used herein the term "interferon activity" refers to antiviral and antigrowth activity characteristic of the interferons. The characteristic antiviral activity of rIFN can be determined using the cytopathic effect inhibition test described by Familletti et al. in Methods in Enzymology 78, 387 (1981). The characteristic antigrowth activity of rIFN can be determined using the procedure described by Evinger, M. & Pestka, S., Methods in Enzymology 79, 45 (1981).

Since the intramolecular disulfide bridge between the cysteine residues at positions 29 and 138 of rIFN- $\alpha$ A is believed to be required for interferon activity, these residues have not to be replaced. Similarly, the cysteine residues at positions 29 and 139 of rIFN- $\alpha$ B through L (and allelic variations thereof) should not be replaced.

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It has been shown by Shepard et al. (Nature 294, 563--565 [1981]) that substitution of the cysteine at position 141 of rIFN- $\beta$  results in a loss of biological activity. Accordingly, this cysteine residue is not to be replaced.

5

In accordance with the present invention the cysteine residue at position 1 of rIFN- $\alpha$ A has been replaced as well as both cysteine residues at positions 1 and 98 of rIFN- $\alpha$ A, without any concomitant loss of interferon activity. Analogously, the cysteine residue(s) at position 1 and/or position 99 (in the case of rIFN- $\alpha$ C through L) and position 1 and/or 100 (in the case of rIFN- $\alpha$ B) can be replaced. In the case of a hybrid rIFN- $\alpha$ , cysteine residues corresponding to these positions, depending upon the parental rIFN and the segment used, may be replaced. In the case of rIFN- $\alpha$ D or a hybrid rIFN- $\alpha$  containing an amino acid sequence of rIFN- $\alpha$ D comprising the cysteine residue at position 87 this cysteine residue may also be replaced.

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The amino acid residues which replace the cysteine residues of the parental interferons in the polypeptides of the invention can be any amino acid residue which is incapable of participating in the formation of an intermolecular disulfide bond. In the case of the N-terminal cysteine residue, i.e. the residue at position 1, this is preferably replaced with glycine. In the case of internal cysteine residues, e.g. the residue at position 98 of rIFN- $\alpha$ A, these are preferably replaced with serine, since the serine side chain most closely resembles the spatial arrangement of the cysteine side chain. Thus, substitution with serine presents the least possibility for disrupting the conformation of the molecule.

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While rIFN- $\alpha$ s, hybrid rIFN- $\alpha$ s, and rIFN- $\gamma$  possess a cysteine residue at position 1, the interferon activity is retained even if this cysteine residue is deleted. Novel polypeptides of this type can be prepared using recombinant

DNA methodology by which the portion of the parental interferon gene which encodes the N-terminus is replaced by a synthetic oligodeoxynucleotide sequence wherein the codon for the first cystein residue is missing in a manner as 5 described in Example 1 hereinafter. In this case the ATG translation initiation signal would immediately be followed by the nucleotide triplet encoding the amino acid residue at position 2 of the parental interferon. The resultant 10 dsDNA can be incorporated into an expression vehicle which can be used to transform a microorganism. The transformant microorganism can then be grown up and under suitable reaction conditions will express the novel polypeptide having 15 interferon activity the amino acid sequence of which is that of a des(Cys 1)-rIFN- $\alpha$ , a hybrid des(Cys 1)-rIFN- $\alpha$  or des(Cys 1)-rIFN- $\gamma$ .

In a preferred embodiment of the invention, a gene 20 encoding rIFN- $\alpha$ A has been modified in a manner described in detail hereinafter and has been expressed to yield a rIFN- $\alpha$ A in which one or both cysteine residues at positions 1 and 98 have been replaced by amino acid residues which are incapable of forming intermolecular disulfide 25 bonds. Accordingly, a preferred embodiment of the present invention is a polypeptide of the formula

25 R<sup>1</sup> ASP LEU PRO GLN THR HIS SER LEU GLY SER ARG ARG  
THR LEU MET LEU LEU ALA GLN MET ARG LYS ILE SER  
LEU PHE SER CYS LEU LYS ASP ARG HIS ASP PHE GLY  
PHE PRO GLN GLU GLU PHE GLY ASN GLN PHE GLN LYS  
ALA GLU THR ILE PRO VAL LEU HIS GLU MET ILE GLN  
30 GLN ILE PHE ASN LEU PHE SER THR LYS ASP SER SER  
ALA ALA TRP ASP GLU THR LEU LEU ASP LYS PHE TYR  
THR GLU LEU TYR GLN GLN LEU ASN ASP LEU GLU ALA -

— R<sup>2</sup> VAL ILE GLN GLY VAL GLY VAL THR GLU THR PRO  
LEU MET LYS GLU ASP SER ILE LEU ALA VAL ARG LYS  
TYR PHE GLN ARG ILE THR LEU TYR LEU LYS GLU LYS  
LYS TYR SER PRO CYS ALA TRP GLU VAL VAL ARG ALA  
5 GLU ILE MET ARG SER PHE SER LEU SER THR ASN LEU  
GLN GLU SER LEU ARG SER LYS GLU

wherein R<sup>1</sup> and R<sup>2</sup> are amino acid residues with the  
10 proviso at least one of R<sup>1</sup> and R<sup>2</sup> is an amino acid  
residue which is incapable of participating in the forma-  
tion of an intermolecular disulfide bond.

Initially, the gene for rIFN- $\alpha$ A has been modified by  
15 excision of a portion of the gene encoding the N-terminus  
of the polypeptide including the nucleotide triplet en-  
coding cysteine at position 1 and replacement with a synthe-  
tic oligodeoxynucleotide coding for glycine at position 1.  
The section of the gene containing the nucleotide triplet  
20 for the cysteine at position 98 remained unchanged. The  
modified gene in a plasmidic expression vehicle, was  
expressed in *E. coli* to produce a polypeptide the amino  
acid sequence of which corresponded to the amino acid  
sequence of rIFN- $\alpha$ A except that it contained a glycine at  
25 position 1 instead of cysteine. The modified polypeptide is  
referred to hereinafter as rIFN- $\alpha$ A (Gly 1). After extrac-  
tion from the cells and purification on a monoclonal anti-  
body immunoaffinity chromatography column, the interferon  
thus obtained was subjected to electrophoresis on sodium  
30 dodecylsulfate polyacrylamide gel (SDS-PAGE). The inter-  
feron separated into a major band, corresponding to mono-  
meric material, and a minor band, corresponding to dimeric  
material. A rIFN- $\alpha$ A expressed from the unmodified  
rIFN- $\alpha$ A gene was electrophoresed on the same gel and  
35 separated into several bands, corresponding to the monomer,  
the dimer and oligomers of higher aggregation, i.e. a tri-  
mer, tetramer, etc.

The product containing a major proportion (more than half) of monomeric rIFN- $\alpha$ A(Gly 1) and a minor proportion of dimeric rIFN- $\alpha$ A(Gly 1) displayed the full antiviral activity characteristics of rIFN- $\alpha$ A on MDBK cells.

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The gene containing the synthetic oligodeoxynucleotide segment encoding glycine at position 1 was then further modified by excision of a segment of the gene including the codon for cysteine at position 98 and replacement with an 10 analogous synthetic oligodeoxynucleotide sequence coding for serine at position 98. This modified gene was expressed in *E. coli* to produce a polypeptide the amino acid sequence of which corresponded to the amino acid sequence of rIFN- $\alpha$ A except that it contained glycine at position 1 15 and serine at position 98. The modified polypeptide is referred to hereinafter as rIFN- $\alpha$ A(Gly 1, Ser 98). After extraction from cells and purification by a monoclonal antibody immunoaffinity chromatography column, the interferon thus obtained was subjected to SDS-PAGE. The interferon migrated as a single band, indicating the presence of 20 essentially only monomeric material.

The product consisting essentially of monomeric rIFN- $\alpha$ A(Gly 1, Ser 98) displayed antiviral activity which 25 was about the same as that of rIFN- $\alpha$ A on MDBK cells.

In a preferred embodiment of this invention, the micro-organisms useful as recipients in the transformation proce-30 dures and unless otherwise noted, are *E. coli* K-12 strain 294 as described in British Patent Publication No. 2055382A (ATCC Accession No. 31446, deposited October 28, 1978) as well as *E. coli* MA210 or RR1 (ATCC Accession No. 31343). 35 However other microbial strains many of which are publicly available or are deposited and available from recognized microorganism depository institutions, such as the American Type Culture Collection (ATCC) can also be used in accordance with the teaching of the present application.

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All recombinant DNA work herein was performed in compliance with applicable guidelines of the National Institutes of Health.

5        The novel polypeptides of the invention can be used for the same purposes as the known interferons in the form of pharmaceutical compositions, e.g. as antiviral and antitumor agents and in the treatment of immunosuppressive conditions. Dosages and dose rates may parallel those currently 10      being used in clinical applications of the known interferons, typically about  $1-200 \times 10^6$  units daily. The polypeptides can be conveniently administered in parenteral dosage form. Suitable dosage forms can be prepared using known formulation methods to prepare pharmaceutically useful 15      compositions wherein the polypeptide is admixed with a pharmaceutically acceptable carrier vehicle. A suitable dosage form will comprise an effective amount of the polypeptide together with a pharmaceutically acceptable carrier vehicle depending upon the particular patient, the mode of 20      administration and the condition to be treated.

BRIEF DESCRIPTION OF THE DRAWINGS

25      Fig. 1 is a representation of the amino acid sequence of rIFN- $\alpha$ A and a nucleotide sequence coding for rIFN- $\alpha$ A.

Fig. 2 is a schematic representation of the process used to produce a dsDNA coding for rIFN- $\alpha$ A(Gly 1).

30      Fig. 3 is a schematic representation of the process used to produce a dsDNA coding for rIFN- $\alpha$ A(Gly 1, Ser 98).

35      Fig. 4 is a schematic representation of the process used to produce a replicable plasmidic expression vehicle for the expression of rIFN- $\alpha$ A(Gly 1).

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Fig. 5 is a schematic representation of the process used to produce a replicable plasmidic expression vehicle for the expression of rIFN- $\alpha$ A(Gly 1, Ser 98).

5 Asterisks in the figures indicate nucleotide substitutions replacing the nucleotide triplets encoding cysteine.

10 In the preferred embodiment of the present invention, the gene sequence encoding rIFN- $\alpha$ A is modified. The double stranded DNA sequence which is the basis for the expression of the modified rIFN- $\alpha$ A consists of a coding strand and a complementary strand, wherein the coding strand, reading from the 5'-end comprises the sequence

15 X GAT CTG CCT CAA ACC CAC AGC CTG GGT AGC AGG AGG  
ACC TTG ATG CTC CTG GCA CAG ATG AGG AAA ATC TCT CTT  
TTC TCC TGC TTG AAG GAC AGA CAT GAC TTT GGA TTT CCC  
CAG GAG GAG TTT GGC AAC CAG TTC CAA AAG GCT GAA ACC  
ATC CCT GTC CTC CAT GAG ATG ATC CAG CAG ATC TTC AAT  
20 CTC TTC AGC ACA AAG GAC TCA TCT GCT GCT TGG GAT GAG  
ACC CTC CTA GAC AAA TTC TAC ACT GAA CTC TAC CAG CAG  
CTG AAT GAC CTG GAA GCC Y GTG ATA CAG GGG GTG GGG  
GTG ACA GAG ACT CCC CTG ATG AAG GAG GAC TCC ATT CTG  
GCT GTG AGG AAA TAC TTC CAA AGA ATC ACT CTC TAT CTG  
25 AAA GAG AAG AAA TAC AGC CCT TGT GCC TGC GAG GTT GTC  
AGA GCA GAA ATC ATG AGA TCT TTT TCT TTG TCA ACA AAC  
TTG CAA GAA AGT TTA AGA AGT AAG GAA

30 wherein X and Y are nucleotide triplets coding for amino acid residues with the proviso that at least one of the amino acid residues for which X and Y are coding is incapable of participating in the formation of an intermolecular disulfide bond.

35 In particular, the present invention provides a method of producing a double stranded DNA sequence encoding a polypeptide having interferon activity which comprises:

(a) cleaving a dsDNA containing a sequence encoding an interferon selected from a rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  with an endonuclease to produce a first dsDNA fragment containing a nucleotide triplet encoding a cysteine residue and one or more other cleavage fragments encoding the remainder of the interferon;

(b) separating the first dsDNA fragment from the other cleavage fragments encoding the remainder of the interferon;

(c) preparing a dsDNA sequence corresponding to the separated first fragment in which, however, the nucleotide triplet coding for the cysteine residue has been replaced by a nucleotide triplet coding for an amino acid residue which is incapable of participating in the formation of an intermolecular disulfide bond, said dsDNA sequence having ends complementary to the ends of the dsDNA sequence(s) encoding the remainder of the interferon; and

(d) ligating said dsDNA sequences in the proper orientation to yield a dsDNA sequence coding for a modified IFN.

The dsDNA encoding an rIFN which is cleaved in step (a) above can be conveniently obtained from an expression or cloning vector containing the IFN structural gene. As a source of dsDNA to serve as a starting material from which to prepare the dsDNA encoding rIFN- $\alpha$ A(Gly 1) and rIFN- $\alpha$ A(Gly 1, Ser 98), plasmid pL 31 was employed. This plasmid, derived from pBR322, contains a 1160 base pair (b.p.) insert encoding a trp promoter-operator, a ribosome binding site, an ATG translation initiation signal, the structural gene encoding the amino acid sequence of rIFN- $\alpha$ A (depicted in Fig. 1) and an untranslated 3'-sequence following the stop codon (Goeddel et al.,

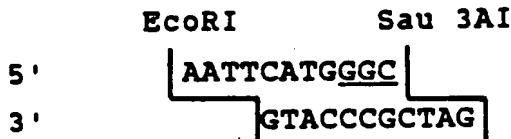
Nature 287, 411 [1980]).

The endonucleases which can be used to cleave the dsDNA in step (a) are well known to those familiar with recombinant DNA technology. Which particular endonuclease is used 5 will depend on the particular rIFN gene involved and the particular cysteine residue(s) to be replaced. It is well within the knowledge of the skilled worker to identify appropriate cleavage sites and to select a suitable endonuclease. The reaction conditions under which the cleavage 10 reactions take place, as well as the methods for separating and purifying the fragments, are well known in the art.

In the preparation of a dsDNA sequence encoding rIFN- $\alpha$ A(Gly 1), advantage was taken of the existence of a 15 Sau 3AI recognition site so that it is possible to cleave the structural gene for rIFN- $\alpha$ A immediately following the nucleotide triplet coding for cysteine at position 1 and to separate this codon from the remainder of the structural gene. 20

The procedure which was employed to produce a dsDNA encoding rIFN- $\alpha$ A(Gly 1) can be described as follows, with specific reference to Fig. 2. The plasmid pL 31, which contained the structural gene for rIFN- $\alpha$ A, was digested 25 completely with Pst I and partially with Sau 3AI. An 854 b.p. fragment was isolated. The coding strand of this fragment, beginning at the Sau 3AI site contained the full DNA sequence encoding rIFN- $\alpha$ A except the TGT codon for cysteine at position 1 and terminated at the Pst I site 360 bases beyond the TGA stop codon. 30

The 5'-end of the rIFN- $\alpha$ A gene thus removed was replaced by ligating the 854 b.p. segment with the synthetic oligodeoxynucleotide 35



5        wherein the GGC-codon coding for glycine replaces the  
TGT-codon coding for the cysteine residue in the unmodified  
rIFN- $\alpha$ A gene. The 865 b.p. dsDNA thus produced contained  
the sequence encoding rIFN- $\alpha$ A(Gly 1) and terminated with  
an Eco RI site at the 5'-end of the coding strand and with  
10      a Pst I site at the other end.

In order to modify the rIFN gene to replace an internal  
cysteine residue, e.g. the cysteine residue at position 98  
of rIFN- $\alpha$ A or IFN- $\alpha$ A(Gly 1), the dsDNA encoding the  
15      rIFN can be cleaved at a first endonuclease cleavage site  
upstream (i.e. toward the 5'-end of the coding strand) of  
the nucleotide triplet encoding the undesired cysteine and  
at a second endonuclease cleavage site downstream (i.e.  
20      toward the 3'-end of the coding strand) of the nucleotide  
triplet encoding the undesired cysteine to isolate a frag-  
ment containing the undesired nucleotide triplet. The frag-  
ment containing the undesired nucleotide triplet is separa-  
ted from the dsDNA encoding the remainder of the rIFN. A  
synthetic dsDNA sequence is prepared which corresponds to  
25      the removed segment except that the nucleotide triplet  
encoding cysteine is replaced by a nucleotide triplet enco-  
ding a different amino acid residue, preferably serine.  
This synthetic dsDNA sequence is then ligated in the proper  
orientation to the dsDNA encoding the remainder of the rIFN.

30      If it is desired to delete two internal cysteine resi-  
dues which are sufficiently close together, this may be  
done by cleaving the gene at a first endonuclease cleavage  
site upstream of the nucleotide triplet encoding the first  
35      undesired cysteine residue and at a second endonuclease  
cleavage site downstream of the nucleotide triplet encoding  
the second undesired cysteine and replacing the segment

thus removed with a corresponding synthetic dsDNA sequence encoding an amino acid sequence in which both cysteines are replaced by different amino acids.

5 With reference to Fig. 3 the following procedure to  
produce a dsDNA encoding rIFN- $\alpha$ A(Gly 1, Ser 98) was used.  
The starting material employed was an 869 b.p. dsDNA enco-  
ding rIFN- $\alpha$ A(Gly 1). This was the 865 b.p. dsDNA produced  
by the previously described procedure to which additional 4  
10 base pairs had been ligated to the 3'-end in order to  
create a terminal Eco RI recognition site useful for inser-  
tion into an expression vector. This dsDNA was cleaved with  
Pvu II, resulting in a 273 b.p. segment containing the  
5'-end of the coding strand for rIFN- $\alpha$ A (Gly 1) and a 596  
15 b.p. segment containing the 3'-end of the coding strand for  
rIFN- $\alpha$ A(Gly 1) including the nucleotide triplet encoding  
cysteine at position 98. The 596 b.p. segment was partially  
digested with Hinf I to produce a 48 b.p. fragment contain-  
ing the nucleotide triplet encoding the cysteine at posi-  
20 tion 98 and a 548 b.p. fragment encoding the remainder of  
the 3'-end. Two synthetic double stranded DNA sequences  
(blocks I and II) were prepared to replace the 48 b.p.  
region of the following sequences:

25 5' CTGAATGACCTGGAAAGCC  
3' GACTTACTGGACCTTCGGTCGCACTAT

Pvu II      Block I

30 Hinf I  
5' AGCGTGATACAGGGGGTGGGGGTGACAGAG  
3' GTCCCCCACCCCCACTGTCTCTGA

36 Block II

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5 The AGC nucleotide triplet encoding serine in block II replaces the TGT triplet encoding cysteine in the deleted 48 b.p. fragment. Block I was ligated to the 273 b.p. fragment and block II was ligated to the 548 b.p. fragment of the original structural gene. The resulting two fragments were then ligated via their 9-base long sticky ends to produce an 869 b.p. dsDNA containing a coding strand which encoded rIFN- $\alpha$ A(Gly 1. Ser 98).

10 The dsDNAs encoding the novel polypeptides of the invention can be incorporated into an expression vehicle which can be used to transform a host microorganism for the purpose of expressing the novel polypeptides. Any of the known and commonly employed expression vectors may be used 15 for this purpose, particularly plasmidic expression vectors.

20 Incorporation of the dsDNA into an expression vector can be achieved in accordance with methods well known in the art. The vector is first linearized by cutting it with an endonuclease at a cleavage site which is appropriately positioned with respect to a promoter-operator sequence 25 directing the expression of the inserted structural gene. The dsDNA encoding the novel polypeptide is then ligated at either end to the cleaved ends of the vector to recirculate the vector. Of course, the ends of the dsDNA insert must be complementary to the cleaved ends of the vector to 30 allow ligation. If necessary, the dsDNA ends can be rendered complementary by building up or cleaving back the ends, using known procedures, provided that the structural gene itself and the associated initiation and termination codons are left intact and that the gene remains in the proper positions and reading frame with respect to the promoter--operator sequence.

35 Accordingly, in accordance with the teachings of the present invention, there is provided a replicable plasmidic expression vehicle comprising:

(a) a replicon;  
(b) a promoter-operator sequence;  
(c) a DNA sequence encoding a ribosome binding site including a translation initiation signal and  
5 (d) a DNA sequence, in phase with said promoter-operator sequence, coding for a polypeptide having interferon activity, said polypeptide comprising the amino acid sequence of an interferon selected from a rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  wherein, however,  
10 at least one cysteine residue has been replaced by an amino acid residue which is incapable of forming intermolecular disulfide bonds, including a translation termination signal.

15 Any of the known plasmidic expression vectors can be employed in the preparation of the replicable plasmidic expression vehicle of this invention. Preferably plasmid pRC 23 can be employed, an expression vector which is derived from pBR 322 and contains the  $P_L$  promoter-operator sequence (Bernard, H.U. and Helinski, D.R., Methods in Enzymology 68, 482). Details concerning the construction of the cloning and expression vector pRC 23 can be obtained from European Patent Application No. 83106730.1. In this vector, the strong  $P_L$  promoter is controlled by the  $\lambda$  cI repressor. The gene coding for the repressor carries a mutation which renders the repressor temperature-sensitive.  
20 At 30°C the repressor functions normally and from about 37°C to about 42°C it is inactivated. Thus the  $P_L$ -promoter is repressed (turned off) at 30°C and depressed (turned on) at 42°C. The ability to control the  $P_L$ -promoter allows one to grow the culture at about 30-36°C without expression of the gene product and then to raise the temperature to about 37-42°C to produce the desired gene product. Plasmid pRC 23 is designed to accept dsDNA sequences  
25 which terminate in Eco RI recognition sites. Accordingly, the 869 b.p. dsDNA sequences previously mentioned, which encode either rIFN- $\alpha$ A(Gly 1) or rIFN- $\alpha$ A(Gly 1, Ser 98)

and terminate in Eco RI recognition sites at either end, can be inserted directly into pRC 23 which has been linearized by cleavage with Eco RI. Ligation is carried out under known conditions using a known ligase, e.g. T4 DNA 5 ligase.

The replicable plasmidic expression vehicle is used to transform a microorganism, preferably E. coli, to produce a transformant which is capable of expressing the polypeptide 10 of the invention. Transformation can be conveniently carried out using known procedures such as treating the host cells with  $\text{CaCl}_2$  at about  $4^\circ\text{C}$ . The transformed microorganism can be grown up under known fermentation conditions and the novel polypeptide expressed in the micro- 15 organism. The polypeptide is then recovered, for example, by lysing the cells and purified by known techniques. Purification can preferably be effected by immunoaffinity chromatography on a column with monoclonal antibodies to the corresponding parental rIFN which are bound to a solid support. A suitable monoclonal antibody which can be used 20 in the purification of rIFN- $\alpha$ A(Gly 1) or rIFN- $\alpha$ A(Gly 1, Ser 98) is described, e.g., by Staehelin et al. in Journal of Biological Chem. 256, 9750 (1981).

25 When the expression vector employed to produce the replicable plasmidic expression vehicle is pRC 23, the resultant transformant microorganism can be grown up to a desired density at a temperature of  $30^\circ\text{C}$  and the temperature can then be raised to about  $42^\circ\text{C}$  to inactivate the 30 repressor protein and initiate expression.

The following examples illustrate but do not limit the present invention. Unless otherwise stated, all parts and percents are by weight and all temperatures are centigrade.

Example 1Preparation of rIFN- $\alpha$ A(Gly 1)5 (a) Preparation of dsDNA Encoding rIFN- $\alpha$ A (Gly 1)

Plasmid pL 31 (6  $\mu$ g) containing the gene encoding rIFN- $\alpha$ A (Nature 287, 411-416 [1980]), was digested completely with Pst I and partially with Sau 3AI. Both digests were performed at 37°C using 20 units Pst I for 10 hr. followed by 5 units of Sau 3AI for 5 min. in a 20  $\mu$ l reaction. The cleavage fragments were separated in a 1.5% agarose gel. A 854 b.p. fragment was isolated which began with the nucleotide triplet encoding Asp at position 2 of the rIFN- $\alpha$ A and terminated at the Pst I site 360 b.p. beyond the translation termination codon.

Using the procedure of Miyoshi et al. (Nucleic Acids Res. 8, 5507-5517 [1980]) the synthetic oligodeoxynucleotides

20

5' AATT~~CATGGGC~~

3' GTACCCGCTAG

were prepared and the 5' ends phosphorylated. The phosphorylated oligodeoxynucleotides were ligated to the Sau 3AI site of the truncated rIFN- $\alpha$ A DNA fragment (100 ng) in a volume of 7  $\mu$ l at 15°C for 16 hours in the presence of T4 ligase. After ligation, the ligase was inactivated by incubation at 70°C for 10 min. and the DNA was digested with 50 units of Eco RI and 10 units of Pst I for 30 2 hours at 37°C in 20  $\mu$ l in order to regenerate the "sticky ends". The resultant 865 b.p. dsDNA fragment, which contained the coding sequence for rIFN- $\alpha$ A(Gly 1), had an Eco RI site at the 5'-end and a Pst I site at the 3'-end of 35 the coding region. The fragment was purified through a 1.5% agarose gel and about 50 ng were recovered for cloning.

(b) Preparation of a Replicable Plasmidic Expression  
Vehicle for Producing rIFN- $\alpha$ A (Gly 1)

Fig. 4 is a schematic representation of the procedure which was employed to prepare the expression vehicle. We employed plasmid pRC 23 as the expression vector to prepare the replicable plasmidic expression vehicle for producing rIFN- $\alpha$ A(Gly 1). As previously indicated, this plasmid was derived from pBR 322 and contained a  $P_L$  promoter-operator. Since pRC 23 was designed to accept genes on an Eco RI restriction site, it was necessary to convert the 3' Pst I site of the 865 b.p. fragment to an Eco RI site. In order to have a sufficient amount of the fragment to perform the modification, we first cloned the 865 b.p. fragment, using plasmid pBR 322, which contains a tetracycline resistance gene, as a cloning vector. We digested 500 ng of pBR 322 to completion at 37°C, with 10 units each of Eco RI and Pst I in a 20  $\mu$ l reaction. The linearized vector thus produced was purified through a 1.0% agarose gel. The 865 b.p. fragment encoding rIFN- $\alpha$ A (Gly 1) (50 ng) was ligated with 100 ng of the linearized pBR 322 using T4 ligase in 10  $\mu$ l for 8 hours at 15°C. After ligation, the mixture was incubated at 70°C for 10 min. and used to transform a competent MC 1061 strain of E. coli cells. The cells were plated out on LB agar containing 10  $\mu$ g/ml of tetracycline and the tetracycline resistant colonies obtained from incubation at 37°C were screened for the presence of the plasmid pBR 322/rIFN- $\alpha$ A (Gly 1). Plasmid screening was performed by the alkaline lysis procedure of Birnboim, H.C. and Doly, J. [Nucleic Acids Res. 7, 1513 (1979)].

Synthetic oligodeoxynucleotides having the sequences

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	Eco RI	Pst I
5'	CGGATCCCGGG	AATTCTGCA
3'	GCCTAGGGCCCTTAAAG	

5B

were prepared by the procedure described by Miyoshi, et al. [Nucleic Acids Res. 8, 5507-5517 (1980)] and phosphorylated with ATP in the presence of polynucleotide kinase at the 5' ends.

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The plasmid pBR 322/rIFN- $\alpha$ A(Gly 1) (200 ng), which contained the 865 b.p. insert encoding rIFN- $\alpha$ A(Gly 1), was digested to completion with 20 units of Pst I at 37°C for 1 hour and the linearized vector was purified through a 10 1.0% agarose gel and recovered from the gel. The synthetic oligodeoxynucleotides (50 ng each) were ligated to 200 ng of the linearized vector in a volume of 5  $\mu$ l at 15°C for 16 hours. After ligase inactivation, the resultant dsDNA was digested with 50 units of Eco RI for 3 hours at 37°C. A 15 869 b.p. fragment encoding rIFN- $\alpha$ A(Gly 1) and terminating in an Eco RI site at either end, was purified on 1.0% agarose gel and recovered for cloning into the Eco RI site of pRC 23.

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About 500 ng of pRC 23 was digested to completion with 10 units of Eco RI at 37°C for 1 hour in 20  $\mu$ l. There was added 1  $\mu$ g of calf intestinal alkaline phosphatase to dephosphorylate the Eco RI ends of the plasmid and incubation was continued for an additional 30 min. The reaction 25 was terminated by heating at 68°C for 10 min. and the linearized vector was purified through a 1.0% agarose gel and recovered. The linearized vector (100 ng) was ligated with 50 ng of the 869 b.p. fragment encoding rIFN- $\alpha$ A(Gly 1) in 10  $\mu$ l at 15°C for 10 hours to produce the replicable 30 plasmidic expression vehicle pRC 23/rIFN- $\alpha$ A(Gly 1).

(c) Preparation of Transformant Containing pRC 23/rIFN- $\alpha$ A (Gly 1)

The ligation mixture was used to transform an RR1 35 strain of *E. coli* cells which contained the compatible plasmid pRK 248 cIts as described by Bernard, H.U. and Helinski, D.R. [Methods in Enzymology, 68, 482]. The compa-

tible plasmid encodes the production of the repressor protein which recognizes and binds the operator portion of the  $P_L$  promoter-operator on pRC 23. The E. coli cells were transformed at 4°C for 30 min. in the presence of 50 mmol 5  $\text{CaCl}_2$ . The cells were plated out at 30°C on LB agar containing ampicillin (50  $\mu\text{g}/\text{ml}$ ). The pRC 23 plasmid contains a gene for ampicillin resistance. After incubation 10 the ampicillin resistant colonies were selected and screened for the presence of the plasmid pRC 23/rIFN- $\alpha\text{A}$  (Gly 1) with the gene inserted in the proper orientation 15 relative to the  $P_L$  promoter.

20 (d) Expression and Purification of rIFN- $\alpha\text{A}$  (Gly 1)

25 A colony of transformants containing the plasmid pRC 23/rIFN- $\alpha\text{A}$ (Gly 1) and the compatible plasmid was grown up in 2 ml of LB agar containing ampicillin (50  $\mu\text{g}/\text{ml}$ ) at a temperature which was not allowed to exceed 30°C. When the  $\text{OD}_{600}$  of the culture reached 0.6, the temperature was raised to 42°C to inactivate the 30 repressor protein and to initiate expression. After 2 hours at 42°C the cells were harvested and lysed in 50  $\mu\text{l}$  of 7M guanidine-HCl at 0°C for 10 min. Extracts were centrifuged for 5 min. at 12,000 g. The supernatants were diluted 1:100 for antiviral assay.

35 25 The rIFN- $\alpha\text{A}$ (Gly 1) was extracted from E. coli cell paste using an extraction buffer containing 2M guanidine-HCl, 2% Triton x 100, 0.1M Tris-Cl, pH 7.5, for 2 hours at 4°C. The extraction mixture was diluted five-fold with cold distilled water, centrifuged at 10,000 x g for 1 hour and purified on a 1 x 2-cm immunoaffinity chromatography column. The immunoaffinity column was packed with 1.0 ml of agarose gel to which there was covalently bound approximately 13 mg of a monoclonal antibody to rIFN- $\alpha\text{A}$ . Preparation of the monoclonal antibody, identified as Li-8, is described by Staehelin et al. in J. Biol. Chem., 256, 9750 (1981).

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The rIFN- $\alpha$ A(Gly 1)-containing extraction buffer (20 ml) was loaded onto the column, which was operated at a flow rate of 1.0 ml/min. or less. The column was then sequentially washed with 5 bed-volumes each of the following solutions

- 5 1. 0.286M guanidine-HCl; 0.286% Triton x-100; 0.1M Tris-Cl, pH 7.5
- 10 2. 0.5M NaCl; 0.2% Triton x-100; 0.025M Tris-Cl, pH 7.5
- 15 3. 1.0M sodium thiocyanate; 0.1% Triton x-100; 0.025M Tris-Cl, pH 7.5
- 20 4. 0.15M NaCl; 0.1% Triton x-100.

The rIFN- $\alpha$ A(Gly 1) was then eluted from the column using an elution solvent of 0.2M acetic acid, 0.15M NaCl and 0.1% Triton x-100 at pH 2.5.

The eluate from the immunoaffinity chromatography column was tested for antiviral activity against vesicular stomatitis virus using the cytopathic effect inhibition test described by Familletti et al., supra. The specific activity of the eluate containing the rIFN- $\alpha$ A(Gly 1) was  $2 \times 10^8$  units/mg of protein, which corresponds to that of rIFN- $\alpha$ A.

Prior to raising the temperature to 42°C to initiate expression, an aliquot of the culture containing the transformants was set aside and stored in glycerol at 30°C. This aliquot was then grown up in a 10-liter fermentor containing LB agar containing ampicillin (50  $\mu$ g/ml) at a temperature not exceeding 30°C until the OD<sub>600</sub> reached 4-5. The temperature was then raised to 42°C to initiate expression. After the OD<sub>600</sub> reached about 12 (2-3 hrs.), the rIFN- $\alpha$ A(Gly 1) was recovered and purified in a manner similar to that described above for the 2-ml culture. The purified rIFN- $\alpha$ A(Gly 1) obtained from the 10-liter culture had an antiviral activity on MDBK cells of  $3.3 \times 10^8$  ( $\pm 0.76 \times 10^8$ ) units/mg of protein.

The purified rIFN- $\alpha$ A(Gly 1) eluate from the immuno-affinity chromatography column was electrophoresed on sodium dodecylsulfate polyacrylamide gel under non-reducing conditions. While a sample of rIFN- $\alpha$ A, displayed bands corresponding to a slow-moving monomer, a fast-moving monomer, a dimer, a trimer and a tetramer a sample of rIFN- $\alpha$ A(Gly 1) displayed only slow-moving monomer and a small amount of dimer. rIFN- $\alpha$ A(Gly 1) which has been neutralized to pH 7.0 shows an increase in the dimer from due to pH-dependent disulfide bond formation between cysteines at position 98. A sample of rIFN- $\alpha$ A(Gly 1) neutralized to pH 7.0 and reduced with  $\beta$ -mercaptoethanol demonstrates that the dimer is a result of disulfide bond formation.

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Example 2Preparation of rIFN- $\alpha$ A (Gly 1, Ser 98)5a) Preparation of dsDNA Encoding rIFN- $\alpha$ A (Gly 1, Ser 98)

20 We prepared the dsDNA encoding rIFN- $\alpha$ A(Gly 1, Ser 98) by taking advantage of a *Pvu* II restriction site upstream of the nucleotide triplet in the rIFN- $\alpha$ A(Gly 1) gene encoding the cysteine at position 98 and a *Hinf* I restriction site downstream of this nucleotide triplet which sites are separated by 48 bases on the coding strand.

25 Referring to Fig. 3, we employed as a starting material pRC 23/rIFN- $\alpha$ A(Gly 1) which contained the 869 b.p. insert of Example 1 encoding rIFN- $\alpha$ A (Gly 1). The 869 b.p. segment was cleaved out of pRC 23 by digesting to completion with 20 units of *Eco* RI and 20 units of *Pvu* II for 2 hours at 37°C in 20  $\mu$ l. The 869 b.p. segment was itself cleaved at the *Pvu* II site just upstream of the nucleotide triplet encoding cysteine at position 98 in two fragments: a 273 b.p. fragment containing the 5'-end of the rIFN- $\alpha$ A(Gly 1) gene and a 596 b.p. segment containing the 3'-end of the gene including the nucleotide triplet encoding cysteine at

position 98. The 596 b.p. fragment was partially digested with 5 units of Hinf I at 37°C for 5 min. in 20  $\mu$ l. Hinf I was then inactivated immediately at 70°C for 10 min. A 548 b.p. Hinf I-Eco RI fragment containing the 3'-end of 5 the rIFN- $\alpha$ A (Gly 1) gene was isolated and purified on 1.5% agarose gel eliminating a 48 b.p. fragment containing the triplet encoding the cysteine at position 98.

Using the procedure of Miyoshi (supra) two double 10 stranded synthetic oligodeoxynucleotides having a complementary overlapping sequence at the 3'-end of the coding strand of the first oligodeoxynucleotide and the 5'-end of the coding strand of the second oligodeoxynucleotide were prepared. When ligated at the complementary sequence, these 15 two oligodeoxynucleotides produced a 48 b.p. insert which was identical to the 48 b.p. fragment cleaved out of the rIFN- $\alpha$ A(Gly 1) gene except that the nucleotide triplet encoding cysteine at position 98 was replaced by an AGC nucleotide triplet encoding serine.

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The two double stranded synthetic oligodeoxynucleotides had the following nucleotide sequences:

25            5'    CTGAATGACCTGGAAGCC  
                  3'    GACTTACTGGACCTTCGGTCGCACTAT

Pvu II      Block I

30            5'    AGCGTGATACAGGGGGTGGGGGTGACAGAG| Hinf I  
                  3'    GTCCCCCACCCCCACTGTCTCTGA|

Block II

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Prior to annealing the individual strands together to produce the two double strands, the 5'-ends were phosphory-

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lated with ATP using polynucleotide kinase to allow ligation. Block I (50 ng) was ligated to the *Pvu* II site of the 273 b.p. cleavage fragment at the 5'-end of the rIFN- $\alpha$ A-(Gly 1) gene (300 ng) for 16 hours at 15°C in a 7  $\mu$ l reaction. Block II (50 ng) was then ligated to the *Hinf* I site of the 548 b.p. cleavage fragment at the 3' end of the rIFN- $\alpha$ A(Gly 1) gene (100 ng) for 16 hours at 15°C in a 7  $\mu$ l reaction. The two dsDNA fragments produced by these ligations were then ligated to each other at the 9 b.p. complementary overlapping portions of Block I and Block II for 16 hours at 15°C. The resultant 869 b.p. dsDNA encoding rIFN- $\alpha$ A(Gly 1, Ser 98) was isolated and purified on a 1.5% agarose gel. The dsDNA had an Eco RI recognition site at either end.

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(b) Preparation of a Replicable Plasmidic Expression Vehicle for Producing rIFN- $\alpha$ A (Gly 1, Ser 98)

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Fig. 5 is a schematic representation of the procedure which was employed to prepare the expression vehicle. Plasmid pRC 23 (500 ng) was digested to completion with 10 units of Eco RI at 37°C for 1 hour in 20  $\mu$ l. There was then added 1  $\mu$ g of calf intestinal alkaline phosphatase and incubation was continued an additional 30 min. The reaction was terminated by heating at 68°C for 10 min. and the linearized vector was purified through a 1.5% agarose gel and recovered. The linearized vector (100 ng) was ligated with 50 ng of the 869 b.p. fragment encoding rIFN- $\alpha$ A-(Gly 1, Ser 98) in 7  $\mu$ l for 10 hours at 15°C to produce the replicable plasmidic expression vehicle pRC 23/rIFN- $\alpha$ A(Gly 1, Ser 98).

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(c) Preparation of Transformant Containing pRC 23/rIFN- $\alpha$ A (Gly 1, Ser 98)

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The ligation mixture was used to transform an RR1 strain of *E. coli* containing the compatible plasmid pRK 248 cts, encoding the production of the temperature-sensitive repressor protein which recognizes and binds the operator

sequence of  $P_L$  promoter-operator on pRC 23. The E. coli cells were transformed at 4°C for 20 min. in the presence of 50 mmol  $\text{CaCl}_2$ . Ampicillin resistant colonies resulting from overnight incubation at 30°C on LB plates were selected and inoculated into 2-ml cultures of LB agar containing ampicillin (50  $\mu\text{g}/\text{ml}$ ). Colonies containing the recombinant plasmid pRC 23/rIFN- $\alpha\text{A}(\text{Gly 1, Ser 98})$  with the gene inserted in the proper orientation relative to the  $P_L$  promoter were selected by screening with the alkaline lysis method of Birnboim (supra).

10 (d) Expression and Purification of rIFN- $\alpha\text{A}(\text{Gly 1, Ser 98})$

15 A colony of transformants containing the plasmid pRC 23/rIFN- $\alpha\text{A}(\text{Gly 1, Ser 98})$  and the compatible plasmid was grown up in 10 liters of LB agar and ampicillin (50  $\mu\text{g}/\text{ml}$ ) at a temperature which was not allowed to exceed 30°C. When the  $\text{OD}_{600}$  of the culture reached 0.6, the temperature was raised to 42°C to inactivate the repressor protein and initiate expression. After 2 hours at 20 42°C the cells were harvested and lysed.

25 The rIFN- $\alpha\text{A}(\text{Gly 1, Ser 98})$  was extracted from E. coli cell paste using 4 volumes of the buffer of Example 1(d) and purified on a 1.6 x 5.0-cm immunoaffinity chromatography column. The immunoaffinity chromatography column was packed with 10 ml of agarose gel to which there was covalently bound approximately 130 mg of a monoclonal antibody to rIFN- $\alpha\text{A}$ . Preparation of the monoclonal antibody, identified as Li-8, is described by Staehelin et al. in J. 30 Biol. Chem., 256, 9750 (1981).

35 The rIFN- $\alpha\text{A}(\text{Gly 1, Ser 98})$ -containing extraction buffer (5,000 ml) was loaded onto the column, which was operated at a flow rate of 5.0 ml/min. or less. The column was then sequentially washed with 5 bed-volumes of the same washing solutions employed in Example 1(d). The rIFN- $\alpha\text{A}(\text{Gly 1, Ser 98})$  was then eluted from the column using the

same elution solvent as in Example 1(d).

The eluate from the immunoaffinity chromatography column was tested for antiviral activity against vesicular 5 stomatitis virus using the cytopathic effect inhibition test described by Familletti (supra). The specific activity of the eluate containing the rIFN- $\alpha$ A(Gly 1, Ser 98) was  $2 \times 10^8$  units/mg of protein, which corresponds to that of rIFN- $\alpha$ A.

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The purified rIFN- $\alpha$ A(Gly 1, Ser 98) eluate from the immunoaffinity chromatography column was electrophoresed on sodium dodecylsulfate polyacrylamide gel under non reducing conditions. While the sample of rIFN- $\alpha$ A(Gly 1, Ser 98) 15 displayed a single band of monomeric interferon a sample of rIFN- $\alpha$ A displayed bands corresponding to the monomer, a dimer, a trimer and higher oligomers.

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CLAIMS:

1. A polypeptide having interferon activity characterized in that its amino acid sequence corresponds to the amino acid sequence of an interferon selected from a rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  wherein, however, at least one cysteine residue has been replaced by an amino acid residue which is incapable of forming an intermolecular disulfide bond.
- 10 2. A polypeptide as claimed in claim 1 which is a rIFN- $\alpha$  or a hybrid rIFN- $\alpha$  in which the cysteine residue at position 1 has been replaced by a glycine residue.
- 15 3. A polypeptide as claimed in claim 2 wherein the cysteine residue at position 98, 99 or 100 has been replaced by a serine residue.
- 20 4. A polypeptide as claimed in claim 2 or 3 wherein said rIFN- $\alpha$  is rIFN- $\alpha$ D or a hybrid rIFN- $\alpha$  containing a fragment of rIFN- $\alpha$ D including the cysteine residue at position 86 and wherein said cysteine residue at position 86 has been replaced by a serine residue.
- 25 5. A polypeptide of the formula  
$$\begin{array}{c} R^1 \text{ ASP LEU PRO GLN THR HIS SER LEU GLY SER ARG ARG} \\ \text{THR LEU MET LEU LEU ALA GLN MET ARG LYS ILE SER} \\ \text{LEU PHE SER CYS LEU LYS ASP ARG HIS ASP PHE GLY} \\ \text{PHE PRO GLN GLU GLU PHE GLY ASN GLN PHE GLN LYS} \\ \text{ALA GLU THR ILE PRO VAL LEU HIS GLU MET ILE GLN} \\ \text{GLN ILE PHE ASN LEU PHE SER THR LYS ASP SER SER} \\ \text{ALA ALA TRP ASP GLU THR LEU LEU ASP LYS PHE TYR} \\ \text{THR GLU LEU TYR GLN GLN LEU ASN ASP LEU GLU ALA -} \end{array}$$

— R<sup>2</sup> VAL ILE GLN GLY VAL GLY VAL THR GLU THR PRO  
LEU MET LYS GLU ASP SER ILE LEU ALA VAL ARG LYS  
TYR PHE GLN ARG ILE THR LEU TYR LEU LYS GLU LYS  
LYS TYR SER PRO CYS ALA TRP GLU VAL VAL ARG ALA  
5 GLU ILE MET ARG SER PHE SER LEU SER THR ASN LEU  
GLN GLU SER LEU ARG SER LYS GLU

wherein R<sup>1</sup> and R<sup>2</sup> are amino acid residues with the  
10 proviso that at least one of R<sup>1</sup> and R<sup>2</sup> is an amino acid  
residue which is incapable of participating in the forma-  
tion of an intermolecular disulfide bond.

6. A polypeptide as claimed in claim 5 wherein R<sup>1</sup> is  
Gly and R<sup>2</sup> is Cys.

15 7. A polypeptide as claimed in claim 5 wherein R<sup>1</sup> is  
Gly and R<sup>2</sup> is Ser.

8. A polypeptide as claimed in claims 1-7 wherein the  
20 first amino acid of the complete sequence is missing.

9. A polypeptide as claimed in claims 1-7 with an addi-  
tional methionine residue as the amino terminus of the  
amino acid sequence.

25 10. Pharmaceutical compositions comprising a major pro-  
portion of a polypeptide with interferon activity, in mono-  
meric form said polypeptide having an amino sequence  
corresponding to that of an interferon selected from a  
30 rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  in which,  
however, at least one cysteine residue has been replaced by  
an amino acid residue which is incapable of forming inter-  
molecular disulfide bonds, said composition being essen-  
tially free of oligomeric forms of the polypeptide of  
35 higher aggregation than the dimer.

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11. A composition as claimed in claim 10 wherein the polypeptide is a polypeptide as claimed in any one of claims 2-9.

5 12. A pharmaceutical composition as claimed in claims 10 or 11 with antiviral activity.

10 13. A double stranded DNA sequence comprising a coding strand and a complementary strand, said coding strand encoding a polypeptide with interferon activity having an amino acid sequence corresponding to that of an interferon selected from a rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  in which, however, at least one cysteine residue has been replaced by an amino acid residue which is incapable of 15 forming intermolecular disulfide bonds.

14. A double stranded DNA sequence as claimed in claim 13 with a coding strand comprising the sequence

20 X GAT CTG CCT CAA ACC CAC AGC CTG GGT AGC AGG AGG  
ACC TTG ATG CTC CTG GCA CAG ATG AGG AAA ATC TCT CTT  
TTC TCC TGC TTG AAG GAC AGA CAT GAC TTT GGA TTT CCC  
CAG GAG GAG TTT GGC AAC CAG TTC CAA AAG GCT GAA ACC  
ATC CCT GTC CTC CAT GAG ATG ATC CAG CAG ATC TTC AAT  
25 CTC TTC AGC ACA AAG GAC TCA TCT GCT GCT TGG GAT GAG  
ACC CTC CTA GAC AAA TTC TAC ACT GAA CTC TAC CAG CAG  
CTG AAT GAC CTG GAA GCC Y GTG ATA CAG GGG GTG GGG  
GTG ACA GAG ACT CCC CTG ATG AAG GAG GAC TCC ATT CTG  
GCT GTG AGG AAA TAC TTC CAA AGA ATC ACT CTC TAT CTG  
30 AAA GAG AAG AAA TAC AGC CCT TGT GCC TGC GAG GTT GTC  
AGA GCA GAA ATC ATG AGA TCT TTT TCT TTG TCA ACA AAC  
TTG CAA GAA AGT TTA AGA AGT AAG GAA

35 wherein X and Y are nucleotide triplets coding for amino acid residues with the proviso that at least one of the amino acid residues for which X and Y are coding is incapable of participating in the formation of an intermolecular

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disulfide bond.

15. A double stranded DNA sequence as claimed in claim  
14, wherein X is a nucleotide triplet encoding a glycine  
5 residue.

16. A double stranded DNA sequence as claimed in claim  
14, wherein X is the nucleotide triplet GGC.

10 17. A double stranded DNA sequence as claimed in claim  
14 or 15, wherein Y is a nucleotide triplet encoding a  
serine residue.

15 18. A double stranded DNA sequence as claimed in claims  
14 to 17, wherein Y is the nucleotide triplet AGC.

19. A replicable plasmidic expression vehicle comprising:

20 (a) a replicon;  
(b) a promoter-operator sequence;  
(c) a DNA sequence encoding a ribosome binding site including a translation initiation signal and  
(d) a DNA sequence, in phase with said promoter-operator  
25 sequence, coding for a polypeptide having interferon activity, said polypeptide comprising the amino acid sequence of an interferon selected from a rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  wherein, however, at least one cysteine residue has been replaced by an  
30 amino acid residue which is incapable of forming intermolecular disulfide bonds, and a translation termination signal.

20. A replicable plasmidic expression vehicle as claimed in claim 19, wherein the polypeptide which is encoded is as claimed in any one of claims 2-8.

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21. A replicable plasmidic expression vehicle as claimed in claim 19, wherein the coding strand of the DNA sequence encoding the polypeptide, reading from the 5' end, comprises a sequence as claimed in any one of claims 14-18.

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22. A transformant microorganism comprising a host microorganism which has been transformed with a replicable plasmidic expression vehicle as claimed in any one of claims 19-21.

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23. A transformant microorganism as claimed in claim 22 wherein the host microorganism is *E. coli*.

24. A process for producing a double stranded (ds) DNA sequence encoding a polypeptide having interferon activity and the amino acid sequence of an interferon selected from a rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  wherein, however, at least one cysteine residue has been replaced by an amino acid residue which is incapable of forming an intermolecular disulfide bond which process comprises

25 (a) cleaving a dsDNA containing a sequence encoding an interferon selected from a rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  with an endonuclease to produce a first dsDNA fragment containing a nucleotide triplet encoding a cysteine residue and one or more other cleavage fragments encoding the remainder of the interferon;

30 (b) separating the first DNA cleavage fragment containing the nucleotide triplet encoding the cysteine from the other cleavage fragments encoding the remainder of the interferon;

35 (c) preparing a dsDNA sequence corresponding to the separated first fragment in which, however, the nucleotide triplet coding for the cysteine residue has been repla-

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ced by a nucleotide triplet coding for an amino acid residue which is incapable of forming an intermolecular disulfide bond, said dsDNA sequence having ends complementary to the ends of the ds DNA sequence(s) encoding the remainder of the interferon; and

5 (d) ligating said dsDNA sequences in the proper orientation to yield a dsDNA coding for said modified interferon.

10 25. A process as claimed in claim 24 wherein the coding strand comprises the sequence

15 X GAT CTG CCT CAA ACC CAC AGC CTG GGT AGC AGG AGG  
ACC TTG ATG CTC CTG GCA CAG ATG AGG AAA ATC TCT CTT  
TTC TCC TGC TTG AAG GAC AGA CAT GAC TTT GGA TTT CCC  
CAG GAG GAG TTT GGC AAC CAG TTC CAA AAG GCT GAA ACC  
ATC CCT GTC CTC CAT GAG ATG ATC CAG CAG ATC TTC AAT  
CTC TTC AGC ACA AAG GAC TCA TCT GCT GCT TGG GAT GAG  
ACC CTC CTA GAC AAA TTC TAC ACT GAA CTC TAC CAG CAG  
20 CTG AAT GAC CTG GAA GCC Y GTG ATA CAG GGG GTG GGG  
GTG ACA GAG ACT CCC CTG ATG AAG GAG GAC TCC ATT CTG  
GCT GTG AGG AAA TAC TTC CAA AGA ATC ACT CTC TAT CTG  
AAA GAG AAG AAA TAC AGC CCT TGT GCC TGC GAG GTT GTC  
AGA GCA GAA ATC ATG AGA TCT TTT TCT TTG TCA ACA AAC  
25 TTG CAA GAA AGT TTA AGA AGT AAG GAA

30 wherein X and Y are nucleotide triplets coding for amino acid residues with the proviso that at least one of the amino acid residues for which X and Y are coding is incapable of participating in the formation of an intermolecular disulfide bond.

35 26. A process as claimed in claim 25 wherein X is a nucleotide triplet encoding a glycine residue.

35 27. A process as claimed in claim 25 wherein X is the nucleotide triplet GGC.

28. A process as claimed in claim 25 or 26 wherein Y is a nucleotide triplet encoding a serine residue.

29. A process as claimed in any one of claims 25 to 28 wherein Y is the nucleotide triplet AGC.

30. A process for the preparation of a polypeptide having interferon activity and the amino acid sequence of an interferon selected from a rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  wherein, however, at least one cysteine residue has been replaced by an amino acid residue which is incapable of forming an intermolecular disulfide bond which process comprises culturing a host transformed by an expression vehicle containing a DNA sequence as defined in any one of claims 25-29, causing the host to express said polypeptide and recovering said polypeptide.

31. A process as claimed in claim 30 wherein said host is E. coli.

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CLAIMS: (austria)

1. A process for producing a double stranded (ds) DNA sequence encoding a polypeptide having interferon activity and the amino acid sequence of an interferon selected from a rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  wherein, however, at least one cysteine residue has been replaced by an amino acid residue which is incapable of forming an intermolecular disulfide bond which process comprises

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(a) cleaving a dsDNA containing a sequence encoding an interferon selected from a rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  with an endonuclease to produce a first dsDNA fragment containing a nucleotide triplet encoding a cysteine residue and one or more other cleavage fragments encoding the remainder of the interferon;

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(b) separating the first DNA cleavage fragment containing the nucleotide triplet encoding the cysteine from the other cleavage fragments encoding the remainder of the interferon;

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(c) preparing a dsDNA sequence corresponding to the separated first fragment in which, however, the nucleotide triplet coding for the cysteine residue has been replaced by a nucleotide triplet coding for an amino acid residue which is incapable of forming an intermolecular disulfide bond, said dsDNA sequence having ends complementary to the ends of the dsDNA sequence(s) encoding the remainder of the interferon; and

(d) ligating said dsDNA sequences in the proper orientation to yield a dsDNA coding for said modified interferon.

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2. A process as claimed in claim 1 wherein the coding strand comprises the sequence

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X GAT CTG CCT CAA ACC CAC CAC AGC CTG GGT AGC AGG AGG  
ACC TTG ATG CTC CTG GCA CAG ATG AGG AAA ATC TCT CTT  
TTC TCC TGC TTG AAG GAC AGA CAT GAC TTT GGA TTT CCC  
CAG GAG GAG TTT GGC AAC CAG TTC CAA AAG GCT GAA ACC  
5 ATC CCT GTC CTC CAT GAG ATG ATC CAG CAG ATC TTC AAT  
CTC TTC AGC ACA AAG GAC TCA TCT GCT GCT TGG GAT GAG  
ACC CTC CTA GAC AAA TTC TAC ACT GAA CTC TAC CAG CAG  
CTG AAT GAC CTG GAA GCC Y GTG ATA CAG GGG GTG GGG  
GTG ACA GAG ACT CCC CTG ATG AAG GAG GAC TCC ATT CTG  
10 GCT GTG AGG AAA TAC TTC CAA AGA ATC ACT CTC TAT CTG  
AAA GAG AAG AAA TAC AGC CCT TGT GCC TGC GAG GTT GTC  
AGA GCA GAA ATC ATG AGA TCT TTT TCT TTG TCA ACA AAC  
TTG CAA GAA AGT TTA AGA AGT AAG GAA

15 wherein X and Y are nucleotide triplets coding for amino acid residues with the proviso that at least one of the amino acid residues for which X and Y are coding is incapable of participating in the formation of an intermolecular disulfide bond.

20 3. A process as claimed in claim 2 wherein X is a nucleotide triplet encoding a glycine residue.

25 4. A process as claimed in claim 2 wherein X is the nucleotide triplet GGC.

5. A process as claimed in claim 2 or 3 wherein Y is a nucleotide triplet encoding a serine residue.

30 6. A process as claimed in any one of claims 2 to 5 wherein Y is the nucleotide triplet AGC.

35 7. A process for the preparation of a polypeptide having interferon activity and the amino acid sequence of an interferon selected from a rIFN- $\alpha$ , a hybrid rIFN- $\alpha$ , rIFN- $\beta$  and rIFN- $\gamma$  wherein, however, at least one cysteine residue has been replaced by an amino acid residue which is

incapable of forming an intermolecular disulfide bond which process comprises culturing a host transformed by an expression vehicle containing a DNA sequence as defined in any one of claims 2-6, causing the host to express said 5 polypeptide and recovering said polypeptide.

8. A process as claimed in claim 7 wherein said host is *E. coli*.

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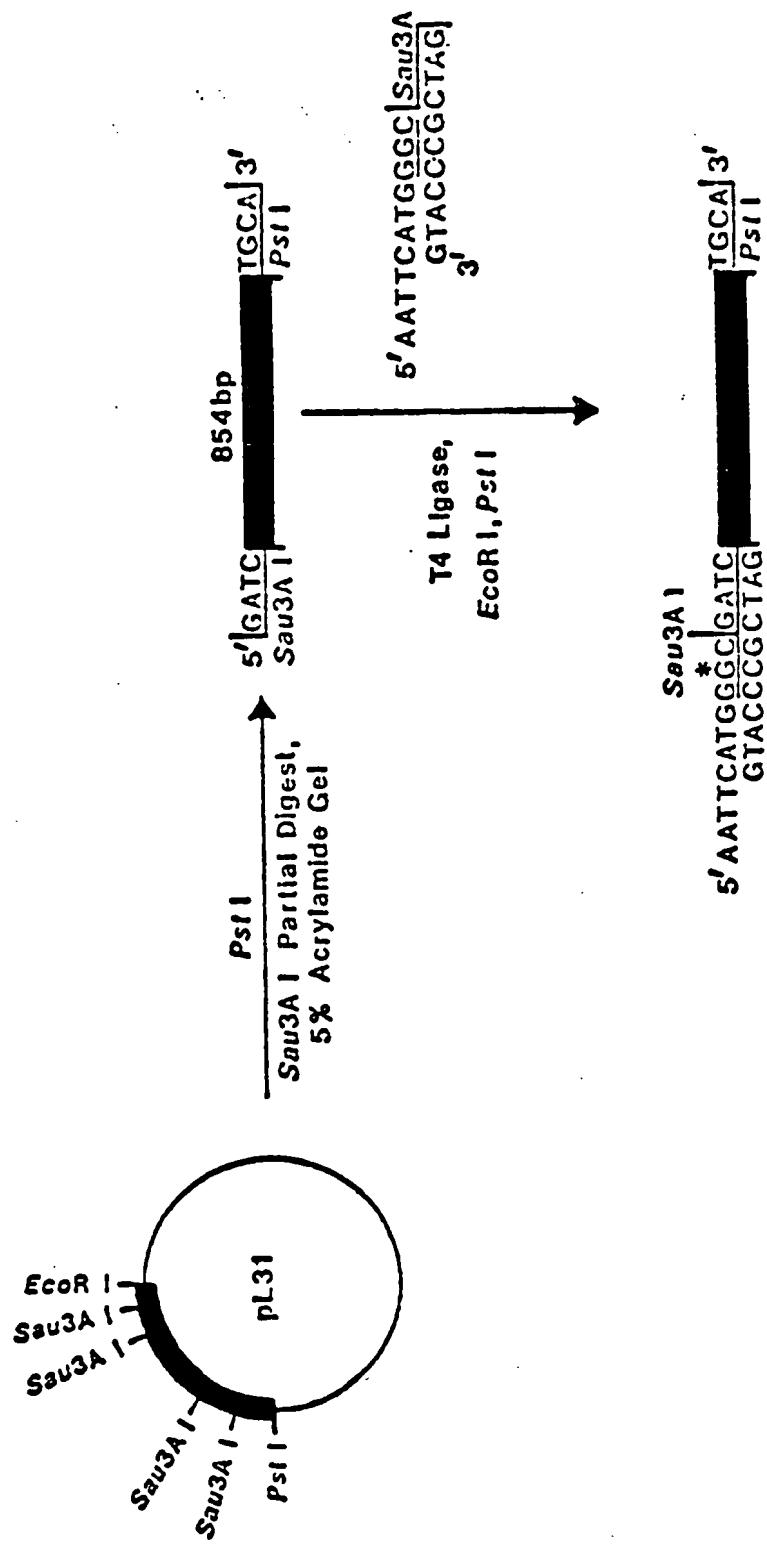
Fig. 1

EcoRI Sau3AI  
 :AATTC ATG TGT :GAT CTG CCT CAA ACC CAC AGC CTG GGT AGC AGG AGG  
 : MET Cys Asp Leu Pro Gln Thr His Ser Leu Gly Ser Arg Arg  
 1 10  
 ACC TTG ATG CTC CTG GCA CAG ATG AGG AAA ATC TCT CTT TTC TCC TGC  
 Thr Leu MET Leu Leu Ala Gln MET Arg Lys Ile Ser Leu Phe Ser Cys  
 20  
 TTG AAG GAC AGA CAT GAC TTT GGA TTT CCC CAG GAG GAG TTT GGC AAC  
 Leu Lys Asp Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Gly Asn  
 30 40  
 CAG TTC CAA AAG GCT GAA ACC ATC CCT GTC CTC CAT GAG ATG ATC CAG  
 Gln Phe Gln Lys Ala Glu Thr Ile Pro Val Leu His Glu MET Ile Gln  
 50 60  
 CAG ATC TTC AAT CTC TTC AGC ACA AAG GAC TCA TCT GCT GCT TGG GAT  
 Gln Ile Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Ala Trp Asp  
 70  
 PvuII  
 GAG ACC CTC CTA GAC AAA TTC TAC ACT GAA CTC TAC CAG :CAG CTG AAT  
 Glu Thr Leu Leu Asp Lys Phe Tyr Thr Glu Leu Thr Gln Gln Leu Asn  
 80 90 HinFI  
 GAC CTG GAA GCC TGT GTG ATA CAG GGG GTG GGG GTG ACA GAG :ACT CCC  
 Asp Leu Glu Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro  
 100  
 CTG ATG AAG GAG GAC TCC ATT CTG GCT GTG AGG AAA TAC TTC CAA AGA  
 Leu MET Lys Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg  
 110 120  
 ATC ACT CTC TAT CTG AAA GAG AAG AAA TAC AGC CCT TGT GCC TGG GAG  
 Ile Thr Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu  
 130 140  
 GTT GTC AGA GCA GAA ATC ATG AGA TCT TTT TCT TTG TCA ACA AAC TTG  
 Val Val Arg Ala Glu Ile MET Arg Ser Phe Ser Leu Ser Thr Asn Leu  
 150  
 CAA GAA AGT TTA AGA AGT AAG GAA TGA  
 Gln Glu Ser Leu Arg Ser Lys Glu  
 160

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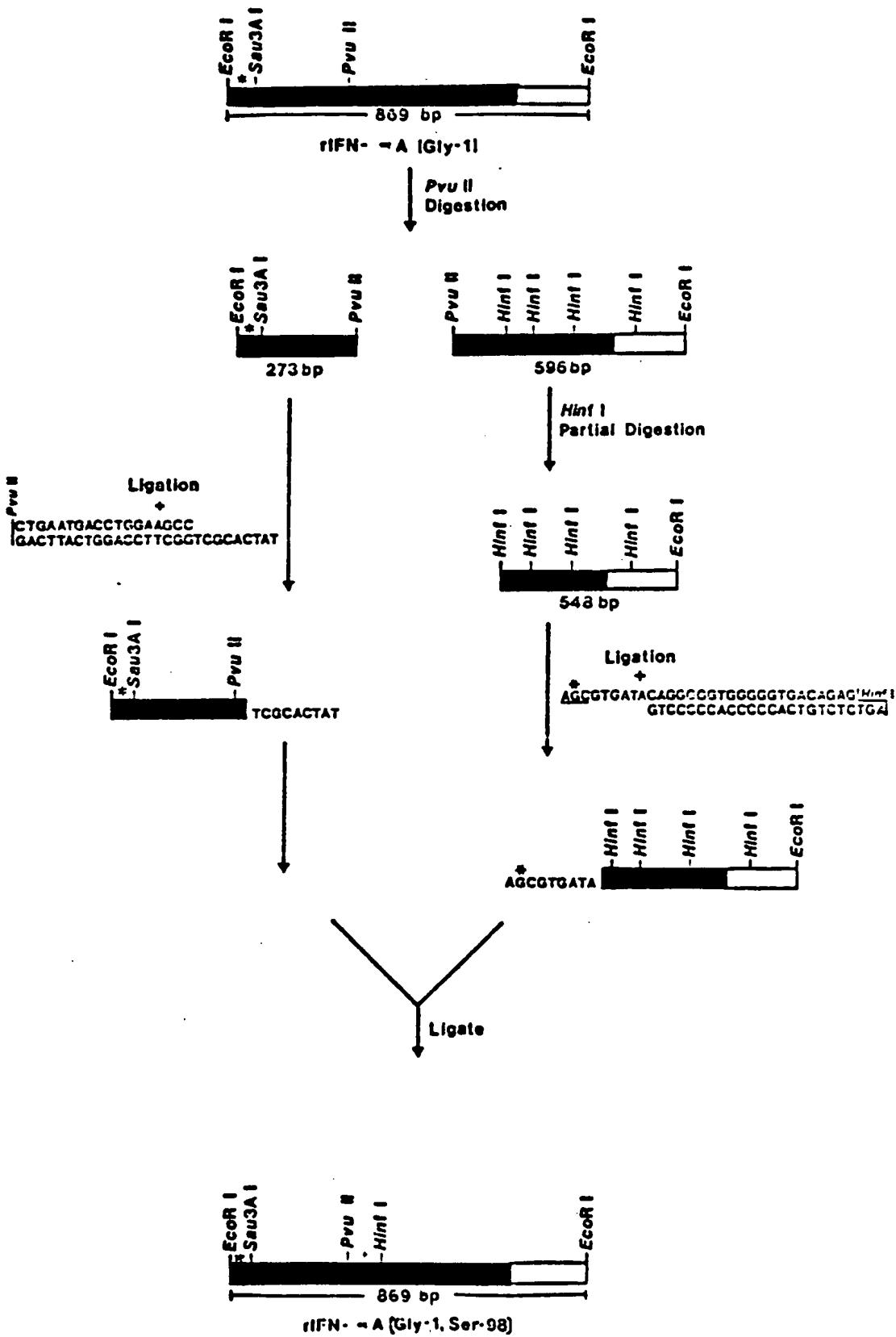
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Fig. 2



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Fig. 3



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Fig. 4

